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(54) Title: IDENTIFICATION AND EXPRESSION OF INSECT STEROID RECEPTOR DNA SEQUENCES

(57) Abstract

The present invention involves the isolation of insect DNA sequences having characteristics of insect steroid receptors. Also described is the putative amino acid sequence for insect steroid receptors as deduced from the DNA sequence.

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Identification and Expression of Insect Steroid Receptor DNA Sequences

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FIELD OF THE INVENTION

This invention relates generally to the use of recombinant DNA methods as applied to the nucleic acid sequences and polypeptides characteristic of insect steroid receptor superfamily members and, more particularly, to uses of such receptors and the DNA regulatory elements associated with genes whose expression they regulate for the production of proteins in cultured cells, and to uses of such hormone receptor proteins and genes in identifying new hormones that control insect development.

BACKGROUND OF THE INVENTION

The temporal sequence of gene expression determines the nature and sequence of steps in the development of the adult animal from the fertilized egg. The common fruit fly, Drosophila melanogaster, provides a favorable model system for studying this genetic control of development. Various aspects of Drosophila development are representative of general insect and, in many respects, vertebrate development.

The steroid hormone 20-OH ecdysone, also known as ß-ecdysone, controls timing of development in many insects. See, generally, Koolman (ed.), Ecdysone: From Chemistry to Mode of Action, Thieme Medical Pub., N.Y. (1989), which is hereby incorporated herein by reference. The generic term "ecdysone" is frequently used as an abbreviation for 20-OH ecdysone. Pulses, or rises and falls, of the ecdysone concentration over a short period

of time in insect development are observed at various stages of Drosophila development.

These stages include embryogenesis, three larval stages and two pupal stages. The last pupal stage ends with the formation of the adult fly. An ecdysone pulse at the end of the third, or last, larval stage pulse triggers the beginning of the metamorphosis of the larva to the adult fly. Certain tissues, called imaginal tissues, are induced to begin their formation of adult structures such as eyes, wings, and legs.

During the larval stages of development, giant polytene chromosomes develop in non-imaginal larval tissues. These cable-like chromosomes consist of aggregates comprising up to about 2,000 chromosomal copies. These chromosome aggregates are extremely useful because they provide a means whereby the position of a given gene within a chromosome can be determined to a very high degree of resolution, several orders of magnitude higher than is typically possible for normal chromosomes.

A "puff" in the polytene chromosomes is a localized expansion or swelling of these cable-like polytene chromosome aggregates that is associated with the transcription of a gene at the puff locus. A puff is, therefore, an indicator of the transcription of a gene located at a particular position in the chromosome.

A genetic regulatory model was proposed to explain the temporal sequence of polytene puffs induced by the ecdysone pulse which triggers the larval-to-adult metamorphosis. See, Ashburner et al., "On the Temporal Control of Puffing Activity in Polytene Chromosomes," Cold Spring Harbor Symp. Quant. Biol. 38:655-662 (1974). This model proposed that ecdysone interacts reversibly with a receptor protein, the ecdysone receptor, to form an ecdysone-receptor complex. This complex would directly induce the transcription of a small set of "early" genes responsible for a half dozen immediately

induced "early" puffs. These early genes are postulated to encode regulatory proteins that induce the transcription of a second set of "late" genes responsible for the formation of the "late" puffs. The model thus defines a genetic regulatory hierarchy of three ranks, the ecdysone-receptor gene in the first rank, the early genes in the second rank, and the late genes in the third. While this model was derived from the puffing pattern observed in a non-imaginal tissue, similar genetic regulatory hierarchies may also determine the metamorphic changes in development of imaginal tissues that are also targets of ecdysone, as well as the changes in tissue development induced by the pulses of ecdysone that occur at other developmental stages.

Various structural data have been derived from receptors for vertebrate steroids and other lipophilic receptor proteins. A "superfamily" of such receptors has been defined on the basis of their structural similarities. See, Evans, "The Steroid and Thyroid Hormone Receptor Superfamily," Science 240:889-895 (1988); Green and Chambon, "Nuclear Receptors Enhance Our Understanding of Transcription Regulation," Trends in Genetics 4:309-314 (1988), both of which are hereby incorporated herein by reference. Where their functions have been defined, these receptors, complexed with their respective hormones, regulate the transcription of their primary target genes, as proposed for the ecdysone receptor in the above model.

Cultivated agriculture has greatly increased efficiency of food production in the world. However, various insect pests exploit cultivated sources of food to their own advantage. These insect pests typically develop by a temporal sequence of events characteristic of their order. Many, including Drosophila, initially develop in a caterpillar or maggot-like larval form. Thereafter, they undergo a metamorphosis from which emerges an adult having characteristic anatomical

features. Anatomic similarity is a reflection of developmental, physiological and biochemical similarities shared by these insects. In particular, the principles governing the role of insect ecdysteroid-hormone receptors in Drosophila development, as described above, likely are shared by many different types of insects.

As one weapon against the destruction of cultivated crops by insects, organic molecules with pesticidal properties are used commonly in attempts to eliminate insect populations. However, the ecological side effects of these pesticides, due in part to their broad activity and lack of specificity, and also in part to the fact that some of these pesticides are not easily biodegradable, significantly affect populations of both insects and other species of animals. Some of these populations may be advantageous from an ecological or other perspective. Furthermore, as the insect populations evolve to minimize the effects of the applied pesticides, greater amounts of pesticides must be applied, causing significant direct and indirect effects on other animals, including humans. Thus, an important need exists for both highly specific and highly active pesticides which are biodegradable. Novel insect hormones which, like the ecdysteroids, act by complexing with insect members of the steroid receptor superfamily to control insect development, are likely candidates for pesticides with these desirable properties.

The use of insect hormones may also have other important applications. Many medically and commercially important proteins can be produced in a usable form by genetically engineered bacteria. However, many expressed proteins are processed incorrectly in bacteria and are preferably produced by genetically engineered eucaryotic cells. Typically, yeast cells or mammalian tissue-culture cells are used. Because it has been observed that protein processing of foreign proteins in yeast cells is also frequently inappropriate, mammalian

cultured cells have become the central focus for the production of many proteins. It is commonly known that the production of large amounts of foreign proteins makes these cells unhealthy, which may affect adversely the yield of the desired protein. This problem may be circumvented, in part, by using an inducible expression system. In such a system, the cells are engineered so that they do not express the foreign protein until an inducing agent is added to the growth medium. In this way, large quantities of healthy cells can be produced and then induced to produce large amounts of the foreign protein. Unfortunately, in the presently available systems, the inducing agents themselves, such as metal ions or high temperature, adversely affect the cells, thus again lowering the yield of the desired foreign protein the cells produce. A need therefore exists for the development of benign inducing factors for efficient production of recombinant proteins. Such factors could also prove invaluable for the therapy of human patients suffering from inability to produce particular proteins, treatment with these factors controlling both the timing and the abundance of the protein produced in the affected individual.

The hormones that complex with mammalian or other vertebrate members of the steroid receptor superfamily are unlikely candidates as such benign factors because they would alter the expression of many target genes in cells bearing these receptors, thereby adversely affecting the host cells.

For these and other reasons, obtaining steroid receptors or nucleic acid information about them has been a goal of researchers for several years. Unfortunately, efforts have been unsuccessful despite a significant investment of resources. The absence of information on the structure and molecular biology of steroid receptors has significantly hindered the ability to produce such products.

Thus, there exists a need for detailed sequence information on insect members of the steroid receptor superfamily, and the genes that encode these receptors and for resulting reagents. Reagents are provided which are useful in finding new molecules which may act as agonists or antagonists of natural insect members of the steroid receptor superfamily, or as components of systems for highly specific regulation of recombinant proteins in mammalian cells.

SUMMARY OF THE INVENTION

In accordance with the present invention, isolated recombinant nucleic acids are provided which, upon expression, are capable of coding for other than a native vertebrate steroid receptor or fragment thereof. These nucleic acids typically comprise a segment having a sequence substantially homologous to one or more coding regions of domains A, B, D, E, or F from an insect steroid receptor superfamily member gene having steroid binding domain homology, e.g., EcR, DHR3, E75A, or E75B. Preferably, the nucleic acids encode a polypeptide capable of binding to a ligand for an insect steroid receptor superfamily member and are capable of hybridizing to an insect steroid receptor superfamily member gene segment under selective hybridization conditions, usually stringent hybridization conditions. Mammalian cells transformed with the nucleic acids are also provided.

In another embodiment, isolated recombinant nucleic acids are included that have sequence exhibiting identity over about 20 nucleotides of a coding segment of an insect steroid receptor superfamily member having steroid binding domain homology. The nucleic acids can be transformed into cells to express a polypeptide which binds to a control lement responsive to a ligand of an insect steroid receptor superfamily.

Alternatively, an isolated DNA molecule is provided comprising a DNA sequence capable of binding to an insect steroid receptor superfamily member other than 20-OH ecdysone receptor, such as DHR3, E75A, or E75B. The DNA sequence will generally be present in an expression vector and promote transcription of an operably linked sequence (e.g., encoding a polypeptide) in response to binding by an insect steroid receptor superfamily member. Cells comprising the nucleic acids are provided, as are cells expressing the polypeptides. In certain embodiments, non-insect cells will be used, including mammalian cells. Also contemplated are recombinant nucleic acids comprising a controlling element responsive to a ligand, e.g., a ligand which binds to an insect steroid receptor superfamily member ligand responsive controlling element, a non-heat shock promoter sequence (e.g., an alcohol dehydrogenase promoter) and a sequence comprising a reporter gene. Usually the controlling element will operate to make transcription of the reporter gene responsive to the presence of the ligand.

Additional embodiments of the present invention include polypeptides comprising an insect steroid receptor superfamily member or fragment thereof, wherein such polypeptide is substantially free of naturally-associated insect cell components and exhibits a biological activity characteristic of an insect steroid receptor superfamily member with a hormone binding domain. Preferably, the insect steroid receptor superfamily member or fragment thereof also comprises a DNA binding domain and the polypeptide is capable of binding to a hormone analogue selected from the group consisting of an insect hormone, an insect hormone agonist and an insect hormone antagonist. The polypeptide can comprise a zinc-finger domain and usually is capable of binding to a DNA controlling element responsive to an insect hormone. As desired, the polypeptide will be fused to a second polypeptide,

typically a heterologous polypeptide which comprises a second steroid receptor superfamily member. Cells, often mammalian cells, comprising the protein are provided.

Fragments of such polypeptides can have a sequence substantially homologous to consensus E1, E2 or E3 region sequences. By way of example, a preferred fragment has a sequence comprising:

a segment at least about 25% homologous to a consensus E1 region sequence;

a segment at least about 30% homologous to a consensus E2 region sequence; and

a segment at least about 30% homologous to a consensus E3 region sequence.

The polypeptides of the present invention have a variety of utilities. For example, a method for selecting DNA sequences capable of being specifically bound by an insect steroid receptor superfamily member can comprise the steps of screening DNA sequences for binding to such polypeptides and selecting DNA sequences exhibiting such binding. Alternatively, methods for selecting ligands, e.g., ecdysteroid analogues, specific for binding to a hormone binding domain of an insect steroid receptor superfamily member can comprise the steps of screening compounds for binding to one or more superfamily members and selecting compounds exhibiting specific binding to the members.

Also included are methods for modulating insect physiology or development (e.g., killing) comprising the steps of screening compounds for binding to an insect steroid receptor superfamily member, selecting compounds exhibiting said binding and administering the ligand to an insect.

Additionally provided are methods for selecting ligands specific for binding to a ligand binding domain of an insect steroid receptor superfamily member comprising combining:

(i) a fusion polypeptide which comprises a ligand binding domain functionally linked to a DNA binding domain of a second steroid receptor superfamily member; and

(ii) a second nucleic acid sequence encoding a second polypeptide, wherein expression of the second nucleic acid sequence is responsive to binding by the DNA binding domain;

screening compounds for an activity of inducing expression of the second polypeptide; and

selecting those compounds which do so.

This will often be performed in a cell, e.g., with cells transformed with DNA encoding a fusion protein. This method allows selction of analogues which are useful in modulating insect physiology or development.

Also provided are methods for producing a polypeptide comprising the steps of:

selecting a cell, typically a mammalian or plant cell which is substantially insensitive to exposure of an insect steroid receptor superfamily ligand;

introducing into said cell:

(i) a receptor for the ligand; and

(ii) a nucleic acid sequence encoding the polypeptide, the nucleic acid sequence operably linked to a controlling element responsive to presence of the selected ligand, wherein a transformed cell is produced; and

exposing the transformed cell to the ligand.

Usually the cell will be a mammalian cell, and will sometimes be introduced into a whole organism, e.g., a plant or animal.

Kits for the determination of expression levels of the nucleic acids and proteins provided herein are made available. Typically, the kit will have at least one compartment which contains a reagent which specifically binds to the desired target molecule, e.g., ligand analogues, receptors, or nucleic acids.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. pMTEcR, a Cu^{2+}-inducible EcR expression plasmid. The P_{MT}, EcR ORF and Act 5c poly A elements are defined in Experimental Example III, part A. The HYG^r ORF confers hygromycin resistance and is under control of the promoter in the LTR of Drosophila transposable elements, copia. The SV40 intron/poly A element provides an intron for a possible splicing requirement, as well as a polyadenylation/cleavage sequence for the HYG^r ORF mRNA. The pAT153 DNA derives from a bacterial plasmid.

Figure 2. The ecdysone-inducible pEcRE/Adh/βgal reporter plasmid. See the text of Experimental Example III, part B, for the construction of this plasmid and the definitions of all symbols (except the SV40 splice and poly A) which are defined in the figure legend.

Figure 3. The constitutive EcR expression plasmid, pActEcR. The construction of this plasmid and the definition of the symbols are given in Experimental Example III, part B.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides novel isolated nucleic acid sequences encoding polypeptide products exhibiting the structure and/or activities of insect members of the steroid receptor superfamily. Having elucidated the structures of these insect steroid receptors from their genes, the separate ligand-binding domains and DNA-binding domains are used individually or in combination to screen for new ligands or DNA sequences which bind to these domains. Thus, for example, by

binding to promotor sequences incorporating a DNA binding site, these receptors will usually control expression of reporter genes for which sensitive assays exist. Or, the hormone-binding domains serve as reagents for screening for agonists or antagonists of steroid receptor superfamily members. Either new classes of molecules, or selected modifications of known ligands will be screened for receptor binding. New ligands obtained in this way find use as highly specific and highly active naturally occurring pesticides. Alternatively, structural information about interactions between the ligand and binding domains directs methods for mutagenizing or substituting particular residues in the binding domains, thereby providing for altered binding specificity. Thus, inter alia, the present invention provides for screening for new ligand molecules, for the design of new ligand-binding domain interactions, for producing novel chimeric steroid receptor superfamily members and for generating new combinations of ligands and binding domains.

The present invention also provides for the isolation or identification of new steroid hormone-responsive elements and associated genes. By appropriate operable linkage of selected sequences to DNA controlling elements which are responsive to binding by the DNA-binding domains of steroid receptor superfamily members, new regulatory combinations result. The present invention further provides for the design of either a binding domain in a member of the insect steroid receptor superfamily that will recognize given DNA sequences, or conversely for the modification of DNA sequences which will bind to particular receptor DNA-binding domains. Both the DNA-binding domain of a superfamily-member polypeptide and its DNA recognition sequence can be coordinately modified to produce wholly new receptor-DNA interactions.

In an alternative embodiment, a DNA-binding sequence recognized by a selected receptor will be operably linked

to a desired genetic sequence for inducible expression. Thus, upon administration of a ligand specific for that selected receptor, the genetic sequence is appropriately regulated. Expression systems are constructed that are responsive to administration of insect steroid receptor superfamily-specific ligands. By identifying and isolating new members of the insect steroid receptor superfamily, new, useful regulatory reagents become available, both hormones and controlling elements.

In another embodiment, highly regulatable expression of a gene is achieved by use of regulatory elements responsive to ligands specific to the superfamily members. If transformed cells are grown under conditions where expression is repressed or not induced, the cells will grow to higher densities and enjoy less stressful conditions. Upon reaching high density, the regulatory ligand molecule is added to cause high expression. Selected cells otherwise insensitive to the inducing ligand will not be affected by exposure to the ligand used to regulate expression. This provides a means both for highly efficient regulatable expression of genes, and for introduction of these genes into intact organisms.

In accordance with specific embodiments of the present invention, nucleic acid sequences encoding portions of insect steroid hormone receptor superfamily members have been elucidated. DNA encoding four different members of the Drosophila steroid receptor superfamily have been characterized: (1) the 20-OH ecdysone receptor, also called the ecdysone receptor (EcR), for which a full-length encoding sequence has been determined; (2) Drosophila hormone receptor 3 (DHR3), a protein with sequence homology to various steroid receptor superfamily members; (3 and 4) E75A and E75B, closely related proteins, encoded by segments of the same gene, and each possessing sequence homology to other steroid receptor superfamily members.

The DNA sequences encoding each of these members of the insect steroid receptor superfamily provide probes for screening for homologous nucleic acid sequences, both in Drosophila and other sources. This screening allows isolation of homologous genes from both vertebrates and invertebrates. Production of large amounts of the encoded proteins is effected by inserting those sequences into expression systems.

The EcR, DHR3, E75A, and E75B genes are each linked to similar DNA sequences which likely function as controlling, or regulatory elements which are responsive to insect steroids. The present invention provides for the isolation of these hormone-responsive control elements, and for their use in regulating gene expression. One embodiment of a DNA construct comprises: (1) multiple copies of an insect steroid receptor superfamily controlling element linked to (2) a minimal gene promoter, preferably not a heat shock gene promoter, which provides highly inducible expression of (3) an operably linked gene. This construct provides a very sensitive assay for the presence of the controlling molecule of the receptor.

Another aspect of the present invention involves cells comprising: (1) isolated recombinant gene segments encoding biologically active fragments of insect steroid receptor superfamily proteins; (2) DNA sequences which bind insect steroid receptors, e.g., the elements involved in hormone-responsive control; or (3) modified receptor proteins. Transformed cells are understood to include their progeny. In particular, the present invention provides for a system whereby expression of polypeptides is responsive to steroid induction. For instance, a system which expresses a desired protein in response to exposure to ecdysone analogues is constructed by operably linking a promoter having an ecdysone-responsive enhancer to a peptide encoding segment.

The present invention also provides insect steroid receptor proteins substantially free from naturally-associated insect cell components. Such receptors will typically be either full-length proteins, functional fragments, or fusion proteins comprising segments from an insect steroid receptor protein fused to a heterologous, or normally non-contiguous, protein domain.

The present invention further provides a number of methods for utilizing the subject receptor proteins. One aspect of the present invention is a method for selecting new hormone analogues. The isolated hormone-binding domains specifically bind hormone ligands, thereby providing a means to screen for new molecules possessing the property of binding with high affinity to the ligand-binding region. Thus, a binding domain of an insect steroid receptor superfamily member will be used as a reagent to develop a binding assay. On one level, the binding domains are useful as affinity reagents for a batch or a column selective process, i.e., to selectively retain ligands which bind. Alternatively, a functional assay is preferred for its greater sensitivity to ligand-binding, whether a direct binding assay or an indirect assay in which binding is linked to an easily assayed function. For example, by operable linkage of an easily assayable reporter gene to a controlling element responsive to binding by an insect steroid receptor superfamily member, in which ligand-binding induces protein synthesis, an extremely sensitive assay for the presence of a ligand or of a receptor results. Such a construct useful for assaying the presence of 20-OH ecdysone is described below. This construct is useful for screening for agonists or antagonists of the 20-OH ecdysone ligand.

In particular, this method allows selecting for ligands which bind to an "orphan" receptor, i.e., a receptor whose ligand is unknown. Binding domains for "unknown" ligands will often originate from either newly

identified insect steroid receptor superfamily members, or from mutagenesis. A hybrid recept r will be created with a ligand-binding domain and DNA-binding domain from different sources. For example, a hybrid receptor between a putative binding domain and a known DNA-binding domain would allow screening for ligands. An "orphan receptor" binding domain will be functionally linked to a known DNA-binding domain which will control a known reporter gene construct whose expression will be easily detected. This system for ligand-receptor binding provides an extremely sensitive assay for ligand-receptor interactions.

Alternatively, the recognition of important features of tertiary structure and spatial interactions between a ligand-binding domain from an insect steroid receptor superfamily member and its ligand will allow selection of new combinations of ligand-binding domains with ligands. Either method provides for selecting unusual ligands which specifically bind a modified polypeptide-binding domain of a receptor. This approach allows selection of novel steroid hormone analogues which exhibit modified specificity for binding to a subgroup of steroid receptors.

The present invention also provides for new and useful combinations of the various related components: the recombinant nucleic acid sequences encoding the polypeptides, the polypeptide sequences, and the DNA sites to which the receptors bind (i.e., the regulatory, or control, elements). For instance, fusing portions of nucleic acid sequences encoding peptides from different sources will provide polypeptides exhibiting hybrid properties, e.g., unusual control and expression characteristics. In particular, hybrid receptors comprising segments from other members of the superfamily, or from other sources, will be made. Combining an insect steroid receptor-responsive enhancer segment with a different polypeptide encoding segment

will produce a steroid-responsive expression system for that polypeptide.

The isolation of insect steroid receptors provides for isolation or screening of new ligands for receptor binding. Some of these will interfere with, or disrupt, normal insect development. These reagents will allow the user to accelerate or decelerate insect development, for instance, in preparing sterile adults for release. Alternatively, a delay or change in the timing of development will often be lethal or will dramatically modify the ability of an insect to affect an agricultural crop. Thus, naturally occurring biodegradable and highly active molecules able to disrupt the timing of insect development will result.

Furthermore, these polypeptides provide the means by which have been raised antibodies possessing specificity for binding to particular steroid receptor classes. Thus, reagents will be produced for determining, qualitatively or quantitatively the presence of these or homologous polypeptides. Alternatively, these antibodies will be used to separate or purify receptor polypeptides.

Transcription sequences of insect steroid receptor superfamily members

The ecdysone receptor gene is a member of the steroid and thyroid hormone receptor gene superfamily, a group of ligand-responsive transcription factors. See, Evans (1988) Science 240:889-895; and Segraves, Molecular and Genetic Analysis of the E75 Ecdysone-Responsive Gene of Drosophila melanogaster (Ph.D. thesis, Stanford University 1988), both of which are hereby incorporated herein by reference for all purposes. These receptors show extensive sequence similarity, especially in their "zinc finger" DNA-binding domains, and also in a ligand (or hormone or steroid) binding domain. Modulation of gene expression apparently occurs in response to binding of a receptor to specific control, or regulatory, DNA

elements. The cloning of receptor cDNAs provides the first opportunity to study the molecular bases of steroid action. The steroid receptor superfamily is a class of receptors which exhibit similar structural and functional features. While the term insect is used herein, it will be recognized that the same methods and molecules will be derived from other species of animals, in particular, those of the class Insecta, or, more broadly, members of the phylum Arthropoda which use ecdysteroids as hormones. Members of the insect steroid receptor superfamily are characterized by functional ligand-binding and DNA binding domains, both of which interact to effect a change in the regulatory state of a gene operably linked to the DNA-binding site of the receptor. Thus, the receptors of the insect steroid receptor superfamily seem to be ligand-responsive transcription factors. The receptors of the present invention exhibit at least a hormone-binding domain characterized by sequence homology to particular regions, designated E1, E2 and E3.

The members of the insect steroid receptor superfamily are typically characterized by structural homology of particular domains, as defined initially in the estrogen receptor. Specifically, a DNA-binding domain, C, and a ligand-binding domain, E, are separated and flanked by additional domains as identified by Krust et al. (1986) EMBO J. 5:891-897, which is hereby incorporated herein by reference.

The C domain, or zinc-finger DNA-binding domain, is usually hydrophilic, having high cysteine, lysine and arginine content -- a sequence suitable for the required tight DNA binding. The E domain is usually hydrophobic and further characterized as regions E1, E2 and E3. The ligand-binding domains of the present invention are typically characterized by having significant homology in sequence and structure to these three regions. Amino proximal to the C domain is a region initially defined as separate A and B domains. Region D separates the more

conserved domains C and E. Region D typically has a hydrophilic region whose predicted secondary structure is rich in turns and coils. The F region is carboxy proximal to the E region (see, Krust et al., supra).

The ligand-binding domain of the members of the insect steroid receptor superfamily is typically carboxyl-proximal, relative to a DNA-binding domain described below. See, Evans (1988) Science 240:889-895. The entire hormone-binding domain is typically between about 200 and 250 amino acids but is potentially shorter. This domain has the subregions of high homology, designated the E1, E2 and E3 regions. See, e.g., Table 4.

The E1 region is 19 amino acids long with a consensus sequence AKX(L/I)PGFXXLT(L/I)(D/E)DQITLL, where X represents any amino acid and the other letters are the standard single-letter code. Positions in parentheses are alternatives. Typically, members of the insect steroid receptor superfamily will have at least about five matches out of the sixteen assigned positions, preferably at least about nine matches, and in more preferred embodiments, at least about ten matches. Alternatively, these insect steroid receptor superfamily members will have homologous sequences exhibiting at least about 25% homology, normally at least about 30% homology, more normally at least about 35% homology, generally at least about 40% homology, more generally at least about 45% homology, typically at least about 50% homology, more typically at least about 55% homology, usually at least about 60% homology, more usually at least about 70% homology, preferably at least about 80% homology, and more preferably at least about 90% homology at positions assigned preferred amino acids.

The E2 region is a 19 amino-acid segment with a consensus sequence:

E(F/Y)(A/V)(L/C)(L/M)KA(I/L)(V/L)L(L/I)
(N/S)(S/P)D(P/-)(R/K)(P/D)GL ,

where - represents an optional absence of an amino acid. Typically, an insect steroid receptor superfamily member will exhibit at least about six matches, preferably at least about eight matches and more preferably at least about nine matches. Alternatively, E2 sequences of insect steroid receptor superfamily members exhibit at least about about 25% homology, normally at least about 30% homology, more normally at least about 35% homology, generally at least about 40% homology, more generally at least about 45% homology, typically at least about 50% homology, more typically at least about 55% homology, usually at least about 60% homology, more usually at least about 70% homology, preferably at least about 80% homology, and more preferably at least about 90% homology at positions assigned preferred amino acids.

The E3 region is a 12 amino-acid segment with a consensus sequence

LXKLLXXLPDLR .

The insect steroid receptor superfamily members will typically show at least about four matches out of the nine assigned preferences in the E3 region, preferably at least about five matches and more preferably at least about six matches. Alternatively, over the assigned positions, members of the insect steroid receptor superfamily will exhibit at least about 25% homology, normally at least about 30% homology, more normally at least about 35% homology, generally at least about 40% homology, more generally at least about 45% homology, typically at least about 50% homology, more typically at least about 55% homology, usually at least about 60% homology, more usually at least about 70% homology, preferably at least about 80% homology, and more preferably at least about 90% homology at positions assigned preferred amino acids.

In preferred embodiments, the insect steroid receptor superfamily members will exhibit matching of at least about five positions in an E1 region, at least

about six positions in an E3 region and at least about four positions in an E3 region. The E1, E2, and E3 regions are defined, e.g., in Table 4.

The DNA-binding domain of these insect steroid receptor superfamily members is characterized by a "zinc fingers" motif. See, Evans (1988) Science 240:889-895. The domain is typically amino proximal to the ligand, or hormone, binding site. Typically, the DNA-binding domain of the insect steroid receptor superfamily members is characterized by clustering of basic residues, a cysteine-rich composition and sequence homology. See, Evans, R. M. (1988) Science 240:889-89; and Experimental section below. Significant polypeptide sequence homology among superfamily members exists. The insect steroid receptor superfamily members will exhibit at least about 30% homology in the 67 ± 1 amino acid region of this domain, normally at least about 40% homology, usually at least about 45% homology, and preferably at least about 55% homology.

Steroids are derivatives of the saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene. Among the molecules in the group "steroids" are: the bile acids, e.g., cholic acid, and deoxycholic acid; the adrenocortical steroids, e.g., corticosterone and aldosterone; the estrogens, e.g., estrone and ß-estradiol; the androgens, e.g., testosterone and progesterone; and the ecdysteroids. The terms steroid or steroid hormones are used interchangeably herein and are intended to include all steroid analogues. Typically, steroid analogues are molecules which have minor modifications of various peripheral chemical groups. See, Koolman (ed.) (1989), cited above, for details on ecdysteroids.

Although ligands for the insect steroid receptor superfamily members have historically been characterized as steroids, the term "steroid" as in "insect steroid receptor superfamily" is not meant only literally. The

use of "steroid" has resulted from a historical designation of members of a group recognized initially to include only molecules having specific defined molecular structures. However, this limitation is no longer applicable since functions are no longer only correlated with precise structures. Thus, there will be members of the insect steroid receptor superfamily, as defined herein, whose ligand-binding specificities are not directed to classically defined "steroids." Typically, the ligands for members of the insect steroid receptor superfamily are lipophilic molecules which are structural analogues of steroid molecules.

The term ligand is meant to refer to the molecules that bind the domain described here as the "hormone-binding domain." Also, a ligand for an insect steroid receptor superfamily member is a ligand which serves either as the natural ligand to which the member binds, or a functional analogue which serves as an agonist or antagonist. The classical definition of "hormone" has been defined functionally by physiologists, see, e.g., Guyton, Textbook of Medical Physiology, Saunders, Philadelphia. The functional term "hormone" is employed because of historic usage, but is meant to apply to other chemical messengers used to communicate between cell types. Recently the distinction between hormones and neurotransmitters has been eroded as various peptide neurotransmitters have been shown to exhibit properties of classically defined hormones. These molecules are typically used in intercellular signal transduction, but are not limited to those molecules having slow or systemic effects, or which act at remote sites.

Substantial homology in the nucleic acid context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 40% of the residues, generally at least about 45%, more generally at least

about 50%, normally at least about 55%, more normally at least about 60%, typically at least about 65%, more typically at least about 70%, usually at least about 75%, more usually at least about 80%, preferably at least about 85%, and more preferably at least about 95% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand or its complement, typically using a sequence derived from Table 1, 2 or 3. Selectivity of hybridization exists when hybridization occurs which is more selective than total lack of specificity. Normally, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 to 25 nucleotides, generally at least about 65%, typically at least about 75%, usually at least about 85%, preferably at least about 90%, and more preferably at least about 95% or more. See, Kanehisa, M. (1984), Nucleic Acids Res. 12:203-213, which is incorporated herein by reference. Stringent hybridization conditions will include salt concentrations of less than about 2.5 M, generally less than about 1.5 M, typically less than about 1 M, usually less than about 500 mM, and preferably less than about 200 mM. Temperature conditions will normally be greater than 20°C, more normally greater than about 25°C, generally greater than about 30°C, more generally greater than about 35°C, typically greater than about 40°C, more typically greater than about 45°C, usually greater than about 50°C, more usually greater than about 55°C, and in particular embodiments will be greater than 60°C, even as high as 80°C or more. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one.

A gene for an insect steroid receptor superfamily member gene includes its upstream (e.g., promoter) and downstream operably linked controlling elements, as well as the complementary strands. See, generally, Watson et al. (1987) The Molecular Biology of the Gene, Benjamin, Menlo Park, which is hereby incorporated herein by reference. A gene geneally also comprises the segment encoding the transcription unit, including both introns and exons. Thus, an isolated gene allows for screening for new steroid receptor genes by probing for genetic sequences which hybridize to either controlling or transcribed segments of a receptor gene of the present invention. Three segments of particular interest are the controlling elements, both upstream and downstream, and segments encoding the DNA-binding segments and the hormone-binding segments. Methods applicable to such screening are analogous to those generally used in hybridization or affinity labeling.

Nucleic acid probes will often be labeled using radioactive or non-radioactive labels, many of which are listed in the section on polypeptide labeling. Standard procedures for nucleic acid labeling are described, e.g., in Sambrook et al. (1989); and Ausubel et al. (1987 and supplements).

Insect steroid receptor superfamily member polypeptides

A polypeptide sequence of the ecdysone receptor is represented in Table 2. Other insect steroid receptor superfamily member polypeptide sequences are set forth in Tables 1 and 3. Preferred nucleic acid sequences of the cDNAs encoding these insect steroid receptor superfamily member polypeptides are also provided in the corresponding tables. Other nucleic acids will be used to encode the proteins, making use of the degeneracy or non-universality of the genetic code.

As used herein, the term "substantially pure" describes a protein or other mat rial, e.g., nucleic

acid, which has been separated from its native contaminants. Typically, a monomeric protein is substantially pure when at least about 60 to 75% of a sample exhibits a single polypeptide backbone. Minor variants or chemical modifications typically share the same polypeptide sequence. Usually a substantially pure protein will comprise over about 85 to 90% of a protein sample, and preferably will be over about 99% pure. Normally, purity is measured on a polyacrylamide gel, with homogeneity determined by staining. Alternatively, for certain purposes high resolution will be necessary and HPLC or a similar means for purification will be used. For most purposes, a simple chromatography column or polyacrylamide gel will be used to determine purity.

The term "substantially free of naturally-associated insect cell components" describes a protein or other material, e.g., nucleic acid, which is separated from the native contaminants which accompany it in its natural insect cell state. Thus, a protein which is chemically synthesized or synthesized in a cellular system different from the insect cell from which it naturally originates will be free from its naturally-associated insect cell components. The term is used to describe insect steroid receptor superfamily members and nucleic acids which have been synthesized in mammalian cells or plant cells, E. coli and other procaryotes.

The present invention also provides for analogues of the insect steroid receptor superfamily members. Such analogues include both modifications to a polypeptide backbone, e.g., insertions and deletions, genetic variants, and mutants of the polypeptides. Modifications include chemical derivatizations of polypeptides, such as acetylations, carboxylations and the like. They also include glycosylation modifications and processing variants of a typical polypeptide. These processing steps specifically include enzymatic modifications, such as ubiquitinylation. See, e.g., Hershko and Ciechanover

(1982), "Mechanisms of Intracellular Protein Breakdown," Ann. Rev. Bioch., 51:335-364.

Other analogues include genetic variants, both natural and induced. Induced mutants are derived from various techniques, e.g., random mutagenesis using reagents such as irradiation or exposure to EMS, or engineered changes using site-specific mutagenesis techniques or other techniques of modern molecular biology. See, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press; and Ausubel et al. (1987 and supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York, each of which is hereby incorporated herein by reference.

As described above, the DNA-binding zinc fingers segment of a receptor shows high specificity of recognition of specific target DNA sequences. An understanding of the DNA-protein binding interactions provides for the modification in a rational manner of either DNA or protein characteristics, or both, to effect specificity of binding for modulation of enhancer activity. More importantly, isolation of genes for new members of the insect steroid receptor superfamily allows their use to produce the receptor polypeptides and to isolate new controlling elements. By using the DNA-binding domains, as described above, controlling elements which are responsive to the ligands bound by the corresponding superfamily members are identified and isolated. This procedure shall yield a variety of controlling elements responsive to ligands. By the methods described above, the ligands for any particular member of the insect steroid receptor superfamily will be identified.

The controlling elements typically are enhancers, but also include silencers or various other types of ligand-r sponsive elements. They usually operate over large distances, but will typically be within about 50 kb, usually within about 35 kb, more usually within

about 20 kb and preferably within about 7 kb of the genes that these elements regulate.

Polypeptide fragments and fusions

Besides substantially full-length polypeptides, the present invention provides for biologically active fragments of the polypeptides. Significant biological activities include ligand-binding, DNA binding, immunological activity and other biological activities characteristic of steroid receptor superfamily members. Immunological activities include both immunogenic function in a target immune system, as well as sharing of immunological epitopes for binding, serving as either a competitor or substitute antigen for a steroid receptor epitope.

For example, ligand-binding or DNA-binding domains from different polypeptides will be exchanged to form different or new fusion polypeptides or fragments. Thus, new chimaeric polypeptides exhibiting new combinations of specificities result from the functional linkage of ligand-binding specificities to DNA-binding domains. This is extremely useful in the design of inducible expression systems.

For immunological purposes, immunogens will sometimes be produced from tandemly repeated polypeptide segments, thereby producing highly antigenic proteins. Alternatively, such polypeptides will serve as highly efficient competitors for specific binding. Production of antibodies to insect steroid receptor superfamily members is described below.

The present invention also provides for other polypeptides comprising fragments of steroid receptor superfamily members. Fusion polypeptides between the steroid receptor segments and other homologous or heterologous proteins are provided, e.g., polypeptide comprising contiguous peptide sequences from different proteins. Homologous polypeptides will often be fusions

between different steroid receptor superfamily members, resulting in, for instance, a hybrid protein exhibiting ligand specificity of one member and DNA-binding specificity of another. Likewise, heterologous fusions, derived from different polypeptides, will be constructed which would exhibit a combination of properties or activities of the parental proteins. Typical examples are fusions of a reporter polypeptide, e.g., luciferase, with another domain of a receptor, e.g., a DNA-binding domain, so that the presence or location of a desired ligand is easily determined. See, e.g., Dull et al., U.S. No. 4,859,609, which is hereby incorporated herein by reference. Other typical gene fusion partners include "zinc finger" segment swapping between DNA-binding proteins, bacterial ß-galactosidase, trpE Protein A, ß-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor. See, e.g., Godowski et al. (1988), Science 241:812-816; and Experimental section below.

Insect steroid receptor superfamily member expression

With the sequence of the receptor polypeptides and the recombinant DNA sequences encoding them, large quantities of members of the insect steroid receptor superfamily will be prepared. By the appropriate expression of vectors in cells, high efficiency protein production will be achieved. Thereafter, standard protein purification methods are available, such as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Deutscher (1990) "Guide to Protein Purification" Methods in Enzymology, vol 182 and others; and Ausubel et al. (1987 and supplements) Current Protocols in Molecular Biology, for techniques typically used for protein purification. Alternatively, in some embodiments high efficiency of production is unnecessary, but the presence of a known inducing protein within a

carefully engineered expression system is quite valuable. For instance, a combination of: (1) a ligand-responsive enhancer of this type operably linked to (2) a desired gene sequence with (3) the corresponding insect steroid receptor superfamily member will be placed together in an expression system provides a specifically inducible expression system. The desired gene sequence will encode a protein of interest, and the corresponding steroid receptor member will often be the ecdysone receptor. Typically, the expression system will be a cell, but in vitro expression systems will also be constructed.

The desired genes will be inserted into any of a wide selection of expression vectors. The selection of an appropriate vector and cell line depends upon the constraints of the desired product. Typical expression vectors are described in Sambrook et al. (1989) and Ausubel et al. (1987 and supplements). Suitable cell lines are available from a depository, such as the ATCC. See, ATCC Catalogue of Cell Lines and Hybridomas (6th ed.) (1988); ATCC Cell Lines, Viruses, and Antisera, each of which is hereby incorporated herein by reference. The vectors are introduced to the desired cells by standard transformation or transfection procedures as described, for instance, in Sambrook et al. (1989).

Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation are described generally, for example, in Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory, which are incorporated herein by reference. Techniques for synthesis of polypeptides are described, for example, in Merrifield, J. Amer. Chem. Soc. 85:2149-2156 (1963).

The recombinant nucleic acid sequences used to produce fusion proteins of the present invention will typically be derived from natural or synthetic sequences.

Many natural gene sequences are obtainable from various cDNA or from genomic libraries using appropriate probes. See, GenBank™, National Institutes of Health. Typical probes for steroid receptors are selected from the sequences of Tables 1, 2 or 3 in accordance with standard procedures. The phosphoramidite method described by Beaucage and Carruthers, Tetra. Letts. 22:1859-1862 (1981) will produce suitable synthetic DNA fragments. A double stranded fragment is then obtainable either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

With the isolated steroid receptor genes, segments of the transcribed segments are available as probes for isolating homologous sequences usually from different sources, e.g., different animals. By selection of the segment used as a probe, particular functionally associated segments will be isolated. Thus, for example, other nucleic acid segments encoding either ligand-binding or DNA-binding domains of new receptors will be isolated. Alternatively, by using steroid-responsive controlling elements as a probe, new steroid-responsive elements will be isolated, along with the associated segment of DNA whose expression is regulated. This method allows for the isolation of ligand-responsive genes, many of which are, themselves, also members of the insect steroid receptor superfamily.

The natural or synthetic DNA fragments coding for a desired steroid receptor fragment will be incorporated into DNA constructs capable of introduction to and expression in an in vitro cell culture. Usually the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria, but alternatively are intended for introduction to, with or without integration into the genome, cultured mammalian or plant or other eucaryotic cell lines. DNA constructs

prepared for introduction into bacteria or yeast will typically include a replication system recognized by the host, the intended DNA fragment encoding the desired receptor polypeptide, transcription and translational initiation regulatory sequences operably linked to the polypeptide encoding segment and transcriptional and translational termination regulatory sequences operably linked to the polypeptide encoding segment. The transcriptional regulatory sequences will typically include a heterologous enhancer or promoter which is recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known. See, Sambrook et al. (1989). Conveniently available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the steroid receptor DNA sequence will generally be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al. (1989); see also, Metzger et al. (1988), Nature 334:31-36.

Genetic constructs

The DNA segments encoding the members of the insect steroid receptor superfamily will typically be utilized in a plasmid vector. In one embodiment an expression control DNA sequence is operably linked to the insect steroid receptor superfamily member coding sequences for expression of the insect steroid receptor superfamily member alone. In a second embodiment an insect steroid receptor superfamily member provides the capability to express another protein in response to the presence of an insect steroid receptor ligand. This latter embodiment is separately described below. The expression control sequences will commonly include eukaryotic enhancer or promoter systems in vectors capable of transforming or

transfecting eucaryotic host cells. Once the vector has been introduced into the appropriate host, the host, depending on the use, will be maintained under conditions suitable for high level expression of the nucleotide sequences.

Steroid-responsive expression of selected genes

For steroid-responsive expression of other genes, the steroid receptor gene will typically be cotransformed with a recombinant construct comprising a desired gene for expression operably linked to the steroid-responsive enhancer or promoter element. In this use, a single expression system will typically comprise a combination of (1) a controlling element responsive to a ligand of an insect steroid receptor superfamily member, (2) a desired gene for expression, operably linked to the controlling element, and (3) an insect steroid receptor superfamily member which can bind to the controlling element. Usually, this system will be employed within a cell, but an in vitro system is also possible. The insect steroid receptor superfamily member will typically be provided by expression of a nucleic acid encoding it, though it need not be expressed at high levels. Thus, in one preferred embodiment, the system will be achieved through cotransformation of a cell with both the regulatable construct and another segment encoding the insect steroid receptor superfamily member. Usually, the controlling element will be an enhancer element, but some elements work to repress expression. In this embodiment, the ligand for the insect steroid receptor superfamily member will be provided or withheld as appropriate for the desired expression properties.

A particularly useful genetic construct comprises an alcohol dehydrogenase promoter operably linked to an easily assayable reporter gene, e.g., ß-galactosidase. In a preferred embodiment of this construct, a multiplicity of copies of the insect steroid receptor

superfamily member is used. For example, operable linkage of controlling elements responsive to insect steroid receptor superfamily members, e,g., EcR, DHR3, E75A and E75B, to the alcohol dehydrogenase (ADH) promoter, or others as described above, and protein coding sequences for a particular reporter protein, as described above, leads to steroid-responsive expression of ß-galactosidase. Such a system provides highly sensitive detection of expression in response to ligand binding, allowing for detection of a productive ligand-receptor interaction.

DNA sequences will normally be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

E. coli is one procaryotic host useful for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as Bacillus subtilis, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species.

Other eucaryotic cells will often be used, including yeast cells, insect tissue culture cells, avian cells, or the like. Preferably, mammalian tissue cell culture will be used to produce the inducible polypeptides of the present invention (see, Winnacker, From Genes to Clones, VCH Publishers, N.Y. (1987), which is incorporated herein by reference). Mammalian cells are preferred cells in which to use the insect steroid receptor superfamily member ligand-responsive gene constructs, because they naturally lack the molecules which confer responses to

the ligands for insect steroid receptor superfamily m mbers.

Mammalian cells are preferred because they are insensitive to many ligands for insect steroid receptor superfamily member. Thus, exposure of these cells to the ligands of the insect steroid receptor superfamily members typically will have negligible physiological or other effects on the cells, or on a whole organism. Therefore, cells can grow and express the desired product, substantially unaffected by the presence of the ligand itself. The ligand will function to cause response either in the positive or negative direction. For example, it is often desirable to grow cells to high density before expression. In a positive induction system, the inducing ligand would be added upon reaching high cell density, but since the ligand itself is benign to the cells, the only physiological imbalances result from the expression, e.g., the product, itself. Alternatively, in a negative repression system, the ligand is supplied until the cells reach a high density. Upon reaching a high density, the ligand would be removed. Introduction of these cells into a whole organism, e.g., a plant or animal, will provide the products of expression to that organism. In this circumstance, the natural insensitivity of cells to the ligands will also be advantageous.

Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferably, the enhancers or promoters will be those naturally associated with genes encoding the steroid receptors, although it will be understood that in many cases others will be equally or more appropriate. Other preferred expr ssion control sequences are enhancers or promoters derived from

viruses, such as SV40, Adenovirus, Bovine Papilloma Virus, and the like.

Similarly, preferred promoters are those found naturally in immunoglobulin-producing cells (see, U.S. Patent No. 4,663,281, which is incorporated herein by reference), but SV40, polyoma virus, cytomegalovirus (human or murine) and the LTR from various retroviruses (such as murine leukemia virus, murine or Rous sarcoma virus and HIV) are also available. See, Enhancers and Eukaryotic Gene Expression, Cold Spring Harbor Press, N.Y., 1983, which is incorporated herein by reference.

The vectors containing the DNA segments of interest (e.g., the steroid receptor gene, the recombinant steroid-responsive gene, or both) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for procaryotic cells, whereas calcium phosphate treatment is often used for other cellular hosts. See, generally, Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), Cold Spring Harbor Press; Ausubel et al. (1987 and supplements) Cement Protocls in Molecular Biology, Greene/Wiley, New York; and Potrykus (1990) "Gene Transfer to Cereals: An Assessment," Bio/Technology 8:535-542, each of which is incorporated herein by reference. Other transformation techniques include electroporation, DEAE-dextran, microprojectile bombardment, lipofection, microinjection, and others. The term "transformed cell" is meant to also include the progeny of a transformed cell.

As with the purified polypeptides, the nucleic acid segments associated with the ligand-binding segment and the DNA-binding segment are particularly useful. These gene segments will be used as probes for screening for new genes exhibiting similar biological activities, though the controlling elements of these genes are of equal importance, as described below.

Many types of proteins are preferentially produced in ucaryotic cell types because of abnormal processing or modification in other cell types. Thus, mammalian proteins are preferably expressed in mammalian cell cultures. Efficient expression of a desired protein is often achieved, as described above, by placing: (1) a desired protein encoding DNA sequence adjacent to controlling elements responsive to ligands for insect steroid receptor superfamily members and an appropriate promoter. Cyclic pulses of ligands in a cell culture may provide periods for cells to recover from effects of production of large amounts of exogenous protein. Upon recovery, the ligand will often be reinduced.

Additional steroid responsive gene elements have also been isolated, e.g., substantially purified, using the techniques of the present invention. Other genes adjacent to, and operably linked to, steroid responsive gene control elements are selectable by locating DNA segments to which steroid receptors specifically bind or by hybridization to homologous controlling elements. For example, other steroid responsive genes have been isolated. Many of the genes which are ligand-responsive will also be new members of the insect steroid receptor superfamily.

Having provided for the substantially pure polypeptides, biologically active fragments thereof and recombinant nucleic acids comprising genes for them, the present invention also provides cells comprising each of them. By appropriate introduction techniques well known in the field, cells comprising them will be produced. See, e.g., Sambrook et al. (1989).

In particular, cells comprising the steroid responsive controlling elements are provided, and operable linkage of standard protein encoding segments to said controlling elements produce steroid responsive systems for gene expression. Cells so produced will often be part of, or be introduced into, intact

organisms, for example, plants, insects (including caterpillars and larvae), and higher animals, e.g., mammals. This provides for regulatable expression of desired genes where the regulating ligand has no other effects on the cells because the cells otherwise lack the receptors and responsive genes. For example, plants will be induced to fruit at desired times by administration of the appropriate ligand, or animals will be ligand-responsive in production of particular products. And, in fact, biochemical deficiencies may be overcome by ligand-responsive expression of cells introduced into an intact organism which, itself, also otherwise lacks genes responsive to the presence of such a ligand. Multiple repeats of the control elements will lead, often, to at least additive or synergistic control. Cells containing these expression systems will be used in gene therapy procedures, including in humans.

Once a sufficient quantity of the desired steroid receptor polypeptide has been obtained, the protein is useful for many purposes. A typical use is the production of antibodies specific for binding to steroid receptors. These antibodies, either polyclonal or monoclonal, will be produced by available in vitro or in vivo techniques.

For production of polyclonal antibodies, an appropriate target immune system is selected, typically a mouse or rabbit. The substantially purified antigen is presented to the immune system in a fashion determined by methods appropriate for the animal and other parameters well known to immunologists. Typical sites for injection are in the footpads, intramuscularly, intraperitoneally, or intradermally. Of course, another species will often be substituted for a mouse or rabbit.

An immunological response is usually assayed with an immunoassay. Normally such immunoassays involve some purification of a source of antigen, for example, produced by the same cells and in the same fashion as the

antigen was produced. The immunoassay will generally be a radioimmunoassay, an enzyme-linked assay (ELISA), a fluorescent assay, or any of many other choices, most of which are functionally equivalent but each will exhibit advantages under specific conditions.

Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 to 10^{10}, or stronger will typically be made by standard procedures as described, e.g., in Harlow and Lane (1988), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory; or Goding (1986), Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York, which are hereby incorporated herein by reference. Briefly, appropriate animals will be selected and the desired immunization protocol followed. After the appropriate period of time, the spleens of such animals are excised and individual spleen cells fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter the cells are clonally separated and the supernatants of each clone are tested for their production of an appropriate antibody specific for the desired region of the antigen.

Other suitable techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse et al., (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281, hereby incorporated herein by reference.

The polypeptides and antibodies of the present invention will be used with or without modification. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors,

inhibitors, fluorescens, chemiluminescers, magnetic particles and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant, chimeric, or humanized, immunoglobulins will be produced, see, e.g., Cabilly, U.S. Patent No. 4,816,567; Jones et al., 1986, Nature 321, 522-526; and published UK patent application No. 8707252; each of which is hereby incorporated herein by reference.

Another use of purified receptor polypeptides is for determination of the structural and biosynthetic aspects of the polypeptides. Structural studies of interactions of the ligand-binding domains with selected ligands are performed by various methods. The preferred method for structural determination is X-ray crystallography but may include various other forms of spectroscopy or chromatography. See, e.g., Connolly, M.L., J. Appl. Crystall., 16:548 (1983); Connolly, M.L., Science 221:709 (1983); and Blundell and Johnson (1976) Protein Crystallography, Academic Press, New York; each of which is hereby incorporated herein by reference. For example, the structure of the interaction between hormone ligand and hormone-binding segments is determined to high resolution. From this information, minor substitutions or modifications to either or both of the ligand and ligand-binding segment are made based upon, e.g., the contact regions between the two. This information enables the generation of modified interactions between a ligand and its binding segment to either increase or decrease affinity of binding and perhaps increase or decrease response to binding. Likewise, the interaction between the zinc finger DNA-binding segments with the specific nucleic acid-binding sequence will be similarly modified.

As a separate and additional approach, isolat d ligand-binding polypeptide domains will be utilized to

screen for new ligands. Binding assays will be developed, analogous, e.g., to immunoassays. This procedure permits screening for new agonists or antagonists of a particular steroid receptor. Isolated DNA-binding segments will be used to screen for new DNA sequences which will specifically bind to a particular receptor-binding segment. Typically, these receptor-specific binding sites will be controlling elements for steroid responsive genes. Thus, having isolated these DNA-binding sequences, genes which are responsive to the binding of a given receptor can be isolated. This provides a method for isolating genes which are responsive to induction or inhibition by a given hormone receptor.

In another aspect of the present invention, means for disrupting insect development are provided where new ligand agonists or antagonists are discovered. These compounds are prime candidates as agonists or antagonists to interfere with normal insect development. By application of new steroid analogues of ligands for insect steroid receptor superfamily members, it is possible to modify the normal temporal sequence of developmental events. For example, accelerating insect development will minimize generation time. This will be very important in circumstances where large numbers of insects are desired finally, for instance, in producing sterile males in Mediterranean fly infestations. Alternatively, it is useful to slow development in some pest infestations, such that the insects reach destructive stages of development only after commercial crops have passed sensitive stages.

In another commercial application, ligands discovered by methods provided by the present invention will be used in the silk-production industry. Here, the silkworms are artificially maintained in a silk-producing larvae stage, thereby being silk productive for extended time periods. The development of larvae will also be

susceptible to acceleration to silk-production in a shorter time peri d than than naturally.

Other analogues of ligands for insect steroid receptor superfamily members will be selected which, upon application, will completely disrupt normal development and, preferably, lead to a lethal result. Slightly modified natural substances will often have greater specificity of action and much higher activities, allowing for lower levels of application. For example, more lipophilic ligands are more readily absorbed directly into the insect surface or cuticle. Thus, extremely low concentrations of natural ligands should be effective in controlling pests. Furthermore, many of these ligands are likely to be relatively easily manufactured, taking advantage of enzymatic production methods. New ligands for insect steroid receptor superfamily members will sometimes be more species specific or will exhibit particularly useful characteristics, for example, being lethal to specific harmful insects. The greater specificity of the hormones will allow avoidance of the use of non-specific pesticides possessing undesired deleterious ecological side effects, e.g., pesticide residue accumulation in food, often having deleterious effects on humans. Furthermore, compounds having structures closely analogous to natural compounds should be susceptible to natural mechanisms of biological degradation.

Another aspect of the present invention provides for the isolation or design of new gene segments which are responsive to ligands for insect steroid receptor superfamily members. For example, use of the nucleic acids to screen for homologous sequences by standard techniques will provide genes having similar structural features. Similarly arranged intron structures will typically be characteristic of larger superfamily categories. The preferred domains for screening will be the ligand-binding or DNA-binding segments, although the

DNA segments which are recognized by the DNA-binding domains, i.e., the controlling elements, will also be of particular interest. Screening for new controlling elements will usually take advantage of known similarities, e.g., sequence homology to other known elements, or homology to the DNA zinc finger-binding domains of other receptors. Receptors and genes important in the general developmental sequence of expression will be discovered. Using this set of developmentally regulated genes will allow selection of particular molecules which are responsible for controlling expression of developmentally regulated genes.

Kits for the determination of expression levels of the nucleic acids and proteins provided herein are made available. Typically, the kit will have at least one compartment which contains a reagent which specifically binds to the desired target molecule, e.g., ligand analogues, receptors, or nucleic acids. These reagents will be used in techniques for assays, e.g., using methods typically used in screening protocols. See, e.g., Sambrook et al. (1989) and Ausubel et al. (1987 and supplements).

The following experimental section is offered by way of example and not by limitation.

EXPERIMENTAL

EXAMPLE I

CLONING STRUCTURE AND EXPRESSION OF THE DROSOPHILA E75 GENE THAT ENCODES TWO MEMBERS OF THE STEROID RECEPTOR SUPERFAMILY.

The following experiments demonstrate that the E75 gene encodes two members of the steroid receptor superfamily. The proteins it encodes share amino acid sequence homology with the conserved DNA-binding and ligand-binding domains of this superfamily. The E75 gene is ecdysone-inducible, and it occupies and causes the

ecdysone-inducible early puff at th 75B locus in the Drosophila polytene chromosome.

A. Cloning of Genomic DNA Encompassing the Ecdysone-Inducible 75B Puff Locus

We have used the method of chromosomal walking (Bender, W., P. Spierer, and D. S. Hogness, 1983. Chromosomal walking and jumping to isolate DNA from the Ace and rosy loci and the Bithorax complex in Drosophila melanogaster. J. Mol. Biol. 168:17-33) to isolate the genomic DNA encompassing the 75B puff region. The starting point for the walk was a genomic clone, designated λ8253, which had been localized by in situ hybridization to the proximal end of 75B. Isolated restriction fragments of λ8253 were used to screen a library of genomic DNA from the Canton S (C^S) strain of D. melanogaster. See (Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstradiatis, 1978, "The isolation of structural genes from libraries of eucaryotic DNA." Cell 15:687-701). Genomic clones λcDm3504 and λcDm3505 were isolated by homology to λ8253.

The walk was then extended in both directions until ~100 kb of genomic DNA had been isolated, and the orientation of the walk was determined by in situ hybridization of the terminal segments to polytene chromosomes. Thereafter, the walk was extended in the rightward direction on the molecular map, or distally relative to the centromere. The 350 kb of genomic DNA encompassed by the walk corresponds to the chromosomal region between bands 75A6-7 and 75B11-13, as determined by in situ hybridization. This region includes the 75B puff, which appears to initiate by simultaneous decondensation of chromosomal bands 75B3-5 and then spreads to surrounding bands.

Methods

Genomic DNA libraries

Canton S genomic DNAs were isolated from a library of sheared, EcoRI-link red Canton S DNA cloned into the Charon 4 λ phage vector. See (Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstradiatis, 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell 15:687-701). O^r genomic DNAs were isolated from a library of sheared DNA, GC-tailed into the sep6 λ vector. See (Meyerowitz, F. M., and D. S. Hogness, 1982. "Molecular organization of a Drosophila puff site that responds to ecdysone." Cell 28:165-176). One step in the chromosomal walk was taken using a cosmid library of SauIIIA partially digested O^r DNA cloned into the cosmid p14B1 by the method of Ish-Horowicz and Burke (Ish-Horowicz, D., and J. F. Burke, 1982. Rapid and efficient cosmid cloning. Nucleic Acids Res. 9:2989-2998).

In situ hybridization

In situ hybridization to polytene chromosomes was carried out with DNA probes that were nick-translated in the presence of ^3H-labeled TTP (NEN), as described by Bonner and Pardue (Bonner, J. J., and M. L. Pardue, 1976. Ecdysone-stimulated RNA synthesis in imaginal discs of Drosophila melanogaster. Assay by in situ hybridization. Chromosoma 58:87-99), with the following modifications: Heat and RNAase treatments of the slides were omitted, and hybridization and washing were at 63°C in 2XSSPE for 18 and 2 hours, respectively.

B. Identification of a 50 kb Region of Cloned Genomic DNA that Contains Sequences Homologous to Ecdysone-induced Transcripts

Restriction fragments of the above genomic clones were tested for their ability to hybridize with each of two cDNA probes, one derived from the RNA in ecdysone-induced cells, and the other from the RNA in noninduced cells. Two differential screens were carried out. In the first, genomic DNA covering the entire 350 kb walk

was examined with cDNA probes synthesized with reverse transcriptase from an oligo(dT) primer annealed to poly(A)+ RNA. The poly(A)+ RNA was prepared from total inner tissues that were mass-isolated from late third instar larvae and incubated in the presence of ecdysone plus cycloheximide, or cycloheximide alone. (See Methods, below. Cycloheximide was included because higher levels of ecdysone-induced transcripts accumulate in its presence.)

Each of the ^{32}P-labeled cDNA probes made from these two poly(A)+ RNAs was applied to one of two duplicate Southern blots that contained, in addition to the genomic fragments from the walk, a control DNA consisting of sequences from the ribosomal protein 49 gene (O'Connell, P., and M. Rosbash, 1984. Sequence, structure and codon preference of the Drosophila ribosomal protein 49 gene. Nucleic Acids Res. 12:5495-5513), which was used to normalize the hybridization intensities of the duplicate blots. This screen revealed sequences specific to ecdysone-induced RNAs only within the λcDm3522 genomic clone that is centered at approximately +220 kb on the molecular map.

Because the above probes will preferentially detect sequences near the 3' termini of the RNAs, particularly in the case of long transcripts, a second differential screen was carried out with cDNA probes primed with random hexamers (see Methods, below). This screen, which was restricted to the 135 kb of genomic DNA between +105 kb and +240 kb, revealed ecdysone-inducible sequences in fragments spread out over an ~50 kb region between +170 kb and +220 kb. This region represents the E75 gene.

Methods

Organ culture and RNA isolation

Late third instar O^r larvae were harvested, washed in 0.7% NaCl, resuspended in Robb's phosphate-buffered saline (PBS) (Robb, J. A., 1968. Maintenance of imaginal

discs of Drosophila melanogaster in chemically defined media. J. Cell. Biol. 41:876-885), preaerated with a blender, and passed through a set of rollers to extrude the organs. This "grindate" was filtered through a coarse Nitex screen to remove carcasses, and settled five times (3-5 minutes per settling) by gravity to remove floating and microscopic debris. Isolated tissues (primarily salivary glands, imaginal discs, gut, and Malphigian tubules) were cultured at 25°C in plastic petri dishes in aerated Robb's PBS. ß-ecdysone (Sigma) (0.2 µl/ml of 10 mg/ml) in ethanol and/or cycloheximide (2 µl/ml of 35 mM) in water was added to the appropriate cultures. Incubations in the presence of cycloheximide were for ~8 hours. Isolated tissues were homogenized in 10 volumes of 6 M guanidine-HCl/0.6 M sodium acetate (pH 5.2), centrifuged at 5000 g for 10 minutes to remove debris, and layered onto a 5.7 M CaCl shelf, as described previously (Chirgwin, J. M., A. E. Przbyla, R. J. MacDonald, and W. J. Rutter, 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299). Poly(A)+ RNA was purified by oligo(dT) chromatography.

Southern blot analysis

Southern blots were performed on nitrocellulose, as described previously (Segraves, W. A., C. Louis, S. Tsubota, P. Schedl, J. M. Rawls, and B. P. Jarry, 1984. The rudimentary locus of Drosophila melanogaster. J. Mol. Biol. 175:1-17). cDNA probes were prepared by reverse transcription (AMV reverse transcriptase; Seikagaku) of 2 µg of poly(A)+ RNA with 700 ng of oligo(dT)$^{12-16}$ (Collaborative Research) or 15 µg of random hexamers (Pharmacia) in a 20 µl reaction mixture containing 80 mM Tris Cl (pH 8.3 at 42°C), 10 mM $MgCl_2$, 100 mM KCl, 0.4 mM DTT, 0.25 mM each of dATP, dGTP, and dTTP, and 100 µCi of [^{32}P]dCTP (800 Ci/mole; Amersham). After incubation at 37°C for 45 minutes, 80 µl of 10 mM

EDTA and 2 μl of 5 N NaOH were added before incubation at 70°C for 10 minutes to denature the products and hydrolyze the RNA. After the addition of 10 μl of 1 M Tris-Cl (pH 7.5) and 5 μl of 1 N HCl, unincorporated label was removed by chromatography on Biogel P60.

C. The E75 Gene Contains Two Overlapping Transcription Units: E75A and E75B

Northern blot analysis of ecdysone-induced and noninduced RNAs, prepared as described above and hybridized with strand-specific DNA probes derived from cloned restriction fragments in the 60 kb region (+166 to +226 kb) containing the E75 gene, demonstrated that this gene produces two classes of ecdysone-inducible mRNAs, both derived from rightward transcription. The E75A class of mRNAs hybridized with probes from both the 5' (left) and 3' (right) ends of the 50 kb E75 gene. The E75B class hybridized only with probes from the 3'-proximal 20 kb of the gene. These results suggest that the A and B classes of ecdysone-inducible RNAs are initiated by different promoters, located about 30 kb apart, and that the two transcription units defined by these promoters overlap in the region downstream from the B promoter.

This suggestion was confirmed by analysis of the structure of cloned cDNAs from the E75A and E75B mRNAs. Approximately 10^6 clones from an early pupal cDNA library (Poole, S. J., L. M. Kauvar, B. Drees, and T. Kornberg, 1985. The engrailed locus of Drosophila: Structural analysis of an embryonic transcript. Cell 40:37-40) were screened at low resolution with genomic DNA probes from the E75 gene region. The 116 cDNA clones identified by this screen were analyzed by restriction digestion and hybridization to a panel of probes derived from the 60 kb (+166 to +226 kb) region. One of the clones, λDm4925, was thereby selected as a representative of the E75A

class of mRNAs, and another, λDm4745, as a representative of the E75B mRNA class.

The genomic regions homologous to these two cDNA clones were further localized by Southern blot analysis, and the nucleotide sequence of these regions and of both cDNA clones was determined. These sequences are given in Table 1, along with those derived from 5' and 3' terminal sequence determinations for each transcription unit. These data demonstrate that the 50 kb E75A transcription unit consists of six exons, labeled in 5' to 3' order: A0, A1, 2, 3, 4 and 5, of which exons A0 and A1 are specific to this unit, while the remaining four are shared with the 20 kb E75B transcription unit. Similarly, the E75B unit contains a specific exon, labeled B1, at its 5' end, which is located just upstream of the shared exon 2. Thus, the E75 gene consists of two transcription units, of which the shorter E75B unit occupies the 3' proximal 20 kb of the longer E75A unit.

Table 1. Sequences of the E75 exons and flanking DNA. The sequence is that of the C^S genomic DNA, which was identical to that of the cDNAs, except for the T→G change indicated at position +2691. This change would convert a leucine to an arginine in the protein sequences. The Dm4925 cDNA extends from just 5' of the EcoRV site at +939 to +4267 in A. The Dm4745 cDNA extends from +804 in B to a point near the HindIII site at +4246 in A. (A) The E75 A exons and flanking DNA. The sequences of the A0, A1, and common exons 2-5 are interrupted by intron sequences (lowercase), which are limited to those near the splice sites and are in agreement with consensus sequences for donor (5') and acceptor (3') splice sites. Negative numbers at the right end of each line refer to the number of base pairs upstream of the E75 A initiation site, positive numbers refer to positions in the E75 A mRNAs, continuing into the 3' flanking DNA. Numbers at the left end of each line refer to amino acid residues in the E75 A protein. The underlined 14 bp sequence at -159 to -172 exhibits a 13/14 bp match to a sequence (CGTAGCGGGTCTC) found 47 bp upstream of the ecdysone-inducible E74 A transcription unit responsible for the early puff at 74EF. This sequence represents the proximal part of a 19 bp sequence in the E74 A promoter that binds the protein encoded by the D. melanogaster zeste gene. Another underlined sequence in the E75 A promoter at -74 to -82 is also found in the E75 B promoter, where it is part of a tandemly repeated octanucleotide (GAGAGAGC) located at -106 to -121 in B. This repeat matches the consensus sequence for the binding sites of the GAGA transcription factor which also binds to the E74 A promoter. Other underlined sequences represent, at -27 to -33, the best match to the TATA box consensus at an appropriate position, three AUG codons that are closely followed by in-frame stop codons in the 5'-leader sequence of the E75 mRNAs, and alternative polyadenylation-cleavage signals at 4591 and 5365 that are used by both E75 A and E75B mRNAs. (B) The B1 exon and its 5'-flanking DNA. The numbering at the right and left ends of the lines follows the same convention as in A. Exons 2-5 shown in A are also used in E75 B, but the amino acid residues and base pair numbers shown in A must be increased by 157 and 375, respectively, to apply to the E75 B protein and mRNA. The first ten nucleotides of the 136-nucleotide E75 B-intron linking the B1 exon to Exon 2 are gtaggttag, whereas the last ten are shown upstream of nucleotide 1178 in A. The underlined sequences represent, in order, the region of homology to a sequence upstream of E75 A, noted above, the best match to the TATA box consensus at -21 to -27, and three AUG codons followed by in-frame stop codons in the 5' leader of the E75 B mRNA.

Panels 1 and 2 are shown in detail in panels 3-8, and 9-10, respectively.

Methods

cDNA libraries

The λDm4925 and λDm4745 cDNAs were isolated from an O^r early pupal cDNA library in λgt10 (Poole, S. J., L. M. Kauvar, B. Drees, and T. Kornberg, 1985. The engrailed locus of Drosophila: structural analysis of an embryonic transcript. Cell 40:37-40). The two cDNAs (λDm4927 and λDm4928) that were used for 3'-end mapping were isolated from an ecdysone-induced salivary gland cDNA library in λ607 prepared by C. W. Jones. (Our strain collection names for the cDNA clones used in these studies are λfDm4925, λfDm4745, λeDm4927, and λeDm4928.)

Northern blot analysis

Probes to be used for Northern blots were cloned into the vector pφX (from R. Mulligan), containing the φX174 origin of replication cloned in between the HindIII and BamHI sites of pBR322. This allowed the synthesis of single-stranded probe DNA (Arai, K., N. Arai, J. Schlomai, and A. Kornberg, 1980. Replication of duplex DNA of phage φX174 reconstituted with purified enzymes. Proc. Natl. Acad. Sci. 77:3322-3326), which was performed by the incubation of supercoiled plasmid DNA with gene A protein, rep and ssb proteins, and DNA polymerase III holoenzyme in a reaction containing 20 mM Tris Cl (pH 7.5), 80 μg/ml BSA, 4% glycerol, 20 mM DTT, 1 mM ATP, 16 mM concentrations of the three unlabeled deoxynucleotides and 1.6 mM concentrations of the labeled deoxynucleotide for 1 hour at 30°C. EDTA was then added to 20 mM, SDS to 0.1%, and proteinase K to 50 μg/ml. The reactions were digested for 30 minutes at 37°C, and unincorporated label was removed by gel filtration.

S1 nuclease protection and primer extension analysis

Single-stranded probes, prepared as described above by the φX in vitro replication system, were purified by electrophoresis on low melting point agarose gels for use

as S1 probes. All other probes were prepared by extension of the -20, 17-mer sequencing primer (New England Biolabs) on single-stranded M13mp (Messing, J., 1983. New M13 vectors for cloning. Methods Enzymol. 101:20-78) or pEMBL (Dente, L., G. Cesareni, and R. Cortes, 1983. pEMBL: A new family of single-stranded plasmids. Nucleic Acids Res. 11:1645-1654) recombinant templates using ^{32}P-labeled nucleotides, followed by cleavage with the appropriate restriction enzyme and purification of the probe on denaturing polyacrylamide gels. Labeled probe (100,000-300,000 cpm) was incubated with 1 μg of poly(A)+ RNA in a 5 μl reaction mixture containing 5 μg of yeast tRNA, 0.4 M NaCl, 40 mM PIPES (pH 6.8), and 1 mM EDTA at 60°C under oil. Reactions were cooled and diluted 1:10 into either S1 digestion or primer extension buffer. S1 nuclease digestions were performed in 50 mM acetate buffer (Na), 400 mM NaCl, and 4 mM $ZnSO_4$ at 20°C for 1 hour with ~15 - 150 Vogt units of S1 nuclease (Boehringer) per 50 μl reaction. Primer extensions were performed at 42°C in 50 mM Tris Cl (pH 8.3 at 42°C), 80 mM KCl, 2 mM DTT, 1 mM of dATP, dCTP, dGTP, and dTTP, with 20 units of AMV reverse transcriptase (Seikagaku) per 50 μl reaction. Reactions were terminated by the addition of EDTA, tRNA carrier was added to the S1 nuclease digestions, and samples were ethanol-precipitated and either electrophoresed directly on 5% or 6% denaturing polyacrylamide gels or glyoxalated (McMaster, G. K., and G. C. Carmichael, 1977. Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. 74:4835-4838) and electrophoresed on 1% agarose gels run in 10 mM sodium phosphate buffer (pH 6.8).

DNA sequence analysis

The cDNA clones λDm4927 and λDm4928 were sequenced by chemical degradation (Maxam, A. M., and W. Gilbert,

1980. Sequencing end-labeled DNA with base-specific chemical cleavage. Methods Enzymol. 65:499-560). All other sequencing was performed using the dideoxynucleotide chain termination method (Sanger, F., A. R. Coulson, B. F. Barrell, A. J. H. Smith, and B. A. Roe, 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. 143:161-178). Fragments were cloned into M13mp vectors (Messing, J., 1983. New M13 vectors for cloning. Methods Enzymol. 101:20-78) or pEMBL (Dente, L., G. Cesareni, and R. Cortes, 1983. pEMBL: A new family of single-stranded plasmids. Nucleic Acids Res. 11:1645-1654) and sequenced directly or following the generation of a set of overlapping deletions using exonuclease III (Henikoff, S., 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359). Sequencing was performed on both strands of the λDm4925 cDNA, the B-specific region of λDm4745 cDNA, the A- and B-specific 5' genomic regions not represented in the cDNAs, and the 3'-flanking region. The remaining exon boundaries of λDm4745 and genomic regions represented within the cDNA clones were sequenced on one strand.

D. The E75 Gene Encodes Two Members of the Steroid Receptor Superfamily

The coding and noncoding sequences of the E75 A and B mRNAs, their splice junctions, and the 5' and 3' flanking sequences are shown in Table 1. Certain sequences of potential interest within the 5' flanking DNA and in the 5' leader mRNA sequences are indicated in the legend to Table 1. We focus here on the large open reading frames of the E75 A and B mRNAs that begin at 380 bp and 284 bp downstream from their respective mRNA start sites, each continuing into the common final exon. The termination codon in exon 5 lies upstream of both alternative polyadenylation sites; thus, the sequence of

the encoded protein is not aff cted by which site is sel cted. Since the open reading frames in the E75 A and B mRNAs begin in the A0 and B1 exons and merge at the beginning of exon 2, the proteins encoded by the two transcription units differ in the amino-terminal region and are the same in the carboxy-terminal region. The specific amino-terminal regions contain 266 and 423 amino acid residues in the E75 A and B proteins, respectively, while their common carboxy-terminal region consists of 971 residues. The predicted molecular weights of the A and B proteins are thus 132,000 and 151,000. The open reading frames display characteristic D. melanogaster codon usage, and their extents have been confirmed by in vitro translation of mRNAs transcribed in vitro from cDNA constructs and by expression of fusion proteins in E. coli. The predicted protein sequence for each protein is punctuated by homopolymeric tracts of amino acids, which are noted in Table 1 and its legend.

Analysis of the sequences of E75 proteins and comparison to the sequences of known proteins have revealed similarity between the E75 proteins and members of the steroid receptor superfamily (Evans, R. M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889-895; Green, S., and P. Chambon, 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends in Genetics 4:309-314). We have used the nomenclature of Krust el al. (Krust, A., S. Green, P. Argos, V. Kumar, P. Walter, J. Bornert, and P. Chambon, 1986. The chicken oestrogen receptor sequence: Homology with v-erbA and the human oestrogen and glucocorticoid receptors. EMBO J. 5:891-897) in dividing the proteins into six regions, A to F, in the amino- to carboxy-terminal direction.

Similarity between E75A and other members of this superfamily is strongest in the C region, a cysteine-lysine-arginine-rich region that is necessary and sufficient for the binding of these receptors to DNA (for

review, see, Evans, R. M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889-895; Green, S., and P. Chambon, 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends in Genetics 4:309-314). The C region consists of 66-68 amino acids, of which 20 residues are invariant within this family. Among these are nine invariant cysteine residues, eight are believed to coordinate zinc in the formation of two zinc finger-like structures (Miller, J., A. D. McLachlan, and A. Klug, 1985. Representative zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J. 4:1609-1614; Freedman, L. P., B. F. Luisi, Z. R. Korszun, R. Basavappa, P. B. Sigler, and K. R. Yamamoto, 1988. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature 334:543-546; Severne, Y., S. Wieland, W. Schaffner, and S. Rusconi, 1988. Metal binding finger structure of the glucocorticoid receptor defined by site-directed mutagenesis. EMBO J. 9:2503-2508). Within the C region, E75A contains all of the highly conserved residues and is approximately as closely related to other members of the steroid receptor superfamily as they are to one another. The closest relative of E75 appears to be the human ear-1 gene, which has nearly 80% amino acid identity to E75 A in the DNA-binding domain.

The other region conserved among members of the steroid receptor superfamily is the E region, which is required for steroid binding and for the linkage of steroid-binding and trans-activation functions (for review, see, Evans, R. M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889-895; Green, S., and P. Chambon, 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends in Genetics 4:309-314). Although overall E-region similarity is clearly significant for the comparison of E75 A to the thyroid hormone, vitamin D, and retinoic

acid receptors, and ear-1, similarity to the glucocorticoid and estrogen receptors is considerably lower. However, the plots of local similarities show a clear similarity to each of these proteins within three subregions of the E region, denoted E1, E2 and E3. The E1 subregion is the most highly conserved and corresponds to a region shown by in vitro mutagenesis to be essential for steroid binding and steroid-dependent trans-activation (Giguere, V., S. M. Hollenberg, M. G. Rosenfield, and R. M. Evans, 1986. Functional domains of the human glucocorticoid receptor. Cell 46:645-652; Danielson, M., J. P. Northrop, J. Jonklaas, and G. M. Ringold, 1987. Domains of the glucocorticoid receptor involved in specific and nonspecific deoxyribonucleic acid binding, hormone activation and transcriptional enhancement. Mol. Endocrinol. 1:816-822). Region E2 is less highly conserved in primary amino acid sequence, but can, in part, be seen as a conserved hydrophobic region in the hydropathy plots of several of these proteins. A deletion of 14 amino acids within this region abolished steroid binding (Rusconi, S., and K. R. Yamamoto, 1987. Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. EMBO J. 6:1309-1315). E3 falls close to the end of the region that is absolutely required for steroid binding.

While the characteristic structural features of the steroid receptor superfamily are well conserved in E75, two novel variations are seen. The first of these concerns the structure of the E75 B protein, which contains a major alteration within its putative DNA-binding domain. The steroid receptor superfamily DNA-binding domain consists of two DNA-binding zinc fingers separated by a less conserved linker region. In E75, as in nearly all other genes of this family, an intron is found between the two fingers. In E75, this splice marks the beginning of the region held in common between the

E75 A and B proteins. This results in the E75 A protein having two fingers, while the E75 B protein has unrelated B-specific sequences in place of the first finger. Other sequences within the B-specific amino-proximal region may contribute to the DNA-binding domain of the E74B protein.

Alternatively, the B protein might bind DNA with only one finger, as GAL4 transcription factor of yeast appears to do. It is possible that these structural differences imply a functional difference in the DNA-binding properties of the E75 A and B proteins that might allow them to differentially regulate the transcription of the late genes that characterize the secondary response to ecdysone in different target tissues.

In this respect, it should be emphasized that the putative hormone- or ligand-binding domain is represented by the E region that is common to the E75A and E75B proteins. Thus, these proteins appear to be receptors for the same hormone, which may regulate the transcription of different sets of genes. These proteins represent "orphan" receptors, in that their hormone, or binding ligand, has not yet been identified. Because ecdysteroids are the only known steroid hormones in Drosophila, the most obvious candidate for an E75 ligand would be ecdysone itself. However, it is unlikely that this is the case, since the putative hormone-binding domain of the E75 proteins does not exhibit the high sequence homology to that of the known Drosophila ecdysone receptor encoded by the EcR gene (see Experimental Example III and Table 2) that would be expected if the E75 proteins were also ecdysone receptors. It, therefore, seems likely that the E75 proteins would bind either a terpenoid juvenile hormone or a novel Drosophila hormone.

The second unusual feature of the E75 proteins is the presence of a large F region, encompassing nearly one half of the proteins. Many of the other receptors have

very small F regions, and no function has yet been ascribed t this region.

Methods

Protein sequence analysis

Sequence data were compiled using the Bionet system. Protein sequence comparison was performed using FASTP (Lipman, D. J., and W. R. Pearson, 1985. Rapid and sensitive protein similarity searches. Science 227:1435-1441) and Bionet IFIND programs.

E. Expression Vectors for E75 Proteins

In order to express the E75 proteins, portions of cDNAs and genomic clones were fused in order to generate cassettes containing the entire E75 A and E75 B protein coding regions. First, BamHI sites were introduced into genomic clones upstream of the initial AUGs of the large open reading frames. Then, E75 A0 exon sequences were fused to sequences of a nearly full-length E75 A cDNA, and E75 B1 exon sequences were fused to sequences of a nearly full-length E75 B cDNA. These cassettes were cloned into pGEM3 (Promega), and transcripts of the open reading frames were prepared using T7 polymerase. These were then translated in the presence of ^{35}S-methionine, and shown to give rise to proteins of appropriate size.

These cassettes have been placed into a variety of expression vectors, including pUCHsneo/Act for expression in Drosophila cells, pSV2 for expression in mammalian cells, and pOTS for expression in bacterial cells.

Methods

BamHI sites were introduced directly upstream of the initial ATGs of the E75A and E75B coding sequence -- at the SspI site upstream of the E75A initial ATG, and at the SacII site upstream of the E75B initial ATG. cDNA and genomic sequences were joined at the EcoRV site in

the A0 exon to construct an E75A cassette, and at the MluI in exon 3 to construct an E75B cassette.

EXAMPLE II

CLONING, STRUCTURE AND EXPRESSION OF THE EcR AND DHR3 GENES THAT ENCODE ADDITIONAL MEMBERS OF THE STEROID RECEPTOR SUPERFAMILY.

The following experiments were carried out after the primary structure of the E75 gene, and of the two members of the steroid receptor superfamily that it encodes, was determined (Experimental, Example I). The purpose of these experiments was to clone and determine the primary structure of other steroid receptor superfamily genes from Drosophila, and of the proteins they encode. The aim was to identify the gene that encodes a Drosophila ecdysone receptor, given that the characteristics of the E75 gene indicated that it did not encode an ecdysone receptor. The first stage of the experimental plan was to use the conserved sequences in the E75A transcription unit that encode the putative DNA-binding domain of the E75A receptor protein as a probe to screen a Drosophila genomic library to identify sequences encoding the putative DNA-binding domains of other Drosophila members of the steroid receptor superfamily. The second stage was to isolate cDNA clones corresponding to the identified genes, as well as additional genomic DNA clones, to obtain the nucleotide sequence of the complete coding region (i.e., the open reading frame encoding the respective receptors) and the exon-intron organization of these genes.

The experiments described below resulted in the cloning and structural characterization of two genes that satisfy the criteria for bona fide members of the steroid receptor superfamily: encoding proteins that exhibit amino acid sequence homology to both the DNA-binding and the hormone-binding domains that are conserved among members of this superfamily. The two genes are called

EcR and DHR3. The EcR gene was originally called DHR23, but was renamed EcR after it was shown to encode an ecdysone receptor (see Experimental Example III). The DHR3 designation stands for Drosophila Hormone Receptor 3.

A. Identification and Chromosomal Mapping of EcR and DHR3 Genomic Clones

Initially, Southern blots of total Drosophila genomic DNA, digested with one or more restriction endonucleases, were probed with a 530 bp fragment of the E75A cDNA containing the sequences encoding the putative DNA-binding domain of the E75A receptor protein (see Experimental Example I) at low and high stringency hybridization conditions.

To isolate the sequences responsible for these low stringency bands, this E75A probe was used to screen a Drosophila genomic library under the same low stringency conditions, counterscreening duplicate filters with E75 intron probes to eliminate phage containing inserts from the E75 gene. Five genome equivalents were screened and 39 non-E75 containing phage were isolated. The 25 most strongly hybridizing clones were divided into six classes on the basis of restriction patterns and cross hybridization, each class containing between one and six independent overlapping genomic inserts.

For each class, a restriction fragment containing the region of hybridization to the E75A probe was localized by Southern blotting. Hybridization of probes derived from these fragments to genomic Southern blots showed that each of the low stringency bands detectable by the E75A probe could be accounted for by one of the six isolated fragments.

The nucleotide sequences of the six restriction fragments were determined to test whether they represent candidate receptor genes. In all cases, DNA sequence similarities with the E75A probe were observed that are sufficient to account for the hybridization of these

fragments with the probe. When the DNA sequences were conceptually translated in all six reading frames, four of the fragments yielded no significant sequence similarity with E75A at the protein level. The remaining two clones, however, showed predicted amino acid sequences with strong similarity to the DNA binding domains of the E75A protein and other steroid superfamily receptors.

These two clones represent the EcR and DHR3 genes, as will become apparent. Probes from these clones were used to map the position of these genes in the polytene chromosomes by in situ hybridization. The EcR and DHR3 chromosomal loci were mapped to positions 42A and 46F, respectively, in the right arm of the second chromosome.

B. Structure of the EcR and DHR3 Genes and Their cDNAs

The DHR3 and EcR genomic clones described above were used to screen a cDNA library prepared from third instar tissues treated with ecdysone and cycloheximide. This procedure allowed the isolation of a large number of cDNA clones, since both genes have a peak period of transcription in late third instar after the rise in ecdysone titer. For each gene, 20 cloned cDNAs were purified and their lengths determined. Restriction maps for the 10 longest cDNAs from each gene were determined and found to be colinear.

For EcR, a 5534 bp cDNA sequence was obtained from two overlapping cDNA clones. It contains an 878 codon open reading frame (ORF) which yields a predicted amino acid sequence expected for a member of the steroid receptor superfamily (Table 2), as described in more detail below. The length of the largest DHR3 cDNA that was isolated (clone DHR3-9) is 4.2 kb. The nucleotide sequence of this cDNA was determined and found to contain a 487 codon AUG-initiated open reading fram (Table 3). As described below, the amino acid sequence of the DHR3

protein predicted from this sequence demonstrates that this prot in is also a bona fide member of the steroid receptor superfamily.

Table 2. Th cDNA sequenc of the EcR gene.

Numerals at the left refer to the nucleotide sequences; those on the right to the amino acid sequence in the EcR protein. Nucleotides 1-5194 are the sequence of EcR-17 cDNA, while nucleotides 5195-5534 derive from the EcR-9 cDNA. The underlined sequences in the 5' and 3' untranslated regions refer, respectively, to the ATG codons and the AATAAA consensus polyadenylation signals. Positions of the introns and the donor and acceptor splice sequences are indicated above the cDNA sequence in small type. The amino acid sequences homologous to the conceived DNA-binding (C region) and hormone-binding (E region) domains of the steroid receptor superfamily are underlined.

Panel 1 is shown in detail in panels 2 and 3.

Table 3. The cDNA sequence of the DHR3 gene. The numbering and underlining of the nucleotide and amino acid sequences hav the same meaning as in Table 2, and the intron positions and donor and acceptor splice sequences are similarly indicated. The sequence of the 5' proximal 2338 nucleotides of the DHR3-9 cDNA is shown. The sequence of the remainder of this 4.2 kb cDNA was determined for only one strand and is not shown. Four silent, third-position differences between the cDNA and genomic DNA sequences are indicated above the cDNA sequence.

The genomic structur of the EcR and DHR3 genes was investigated by isolating additional genomic DNA clones that form overlapping sets that contain all of the sequences found in the respective cDNA clones. The exons contained in these cDNAs were mapped within the genomic DNA by comparison of cDNA and genomic clones via Southern blot analysis, mapping of restriction cleavage sites, and finally, by determination of the nucleotide sequence of the genomic DNA in regions that contain the exon/intron boundaries. Table 2 and 3 show these boundaries and the sequence of the splice junctions for the EcR and DHR3 genes, respectively. All of these splice junctions conform to the splice donor and acceptor consensus sequences.

For EcR, the cDNA sequence shown in Table 2 is split into six exons spread over 36 kb of genomic DNA, with the ORF beginning in the second exon and ending in the sixth. For DHR3, the cDNA sequence derives from nine exons spread over 18 kb, with the ORF beginning in the first exon and ending in the ninth. Because the 5' and 3' ends of the respective mRNAs were not mapped, it should be emphasized that these genes may have additional noncoding exons at their 5' or 3' ends.

The EcR and DHR3 gene structures differ significantly from those of all previously examined steroid receptor superfamily genes. Comparison with the genes for 11 other receptor homologues for which at least partial structural information is available reveals that the positions of certain exon boundaries have been conserved in evolution. This conservation is most striking in the portion of the genes encoding DNA-binding domains. In the nine other cases where the structure of this region has been examined, the two halves of the DNA-binding domain are always encoded by separate exons. If we exclude the Drosophila genes knirps, knirps-related, and egon (which are not bona fide receptor homologues since they lack the hormone-binding domain sequence

similarity), these are always small exons, the second one invariably ending in the fourth codon beyond the conserved Met codon at the end of the C region. Thus, these exons each encode one of the two predicted zinc fingers of the DNA-binding domain. In contrast, both zinc fingers of the putative DNA-binding domain of the EcR and DHR3 receptors are encoded by a single exon. It is possible that our screen specifically selected for genes lacking the above intron. The screen selected genomic clones that hybridize to an E75A cDNA probe that, of course, lacks this intron. Genomic sequences containing a contiguous sequence encoding the DNA-binding domain would be expected to hybridize to this probe better than clones from genes containing the intron. This would explain the successful isolation of the EcR and DHR3 genes, and the failure to isolate the genes of other Drosophila members of the steroid receptor superfamily.

Methods

Isolation of cDNA and additional genomic clones

Subclones of the originally isolated DHR3 and EcR genomic clones were used to screen a cDNA library prepared from third instar tissues treated with ecdysone and cycloheximide. This library was chosen because both genes are relatively highly expressed at the end of third instar, and because of the high quality of the library. Of the 270,000 primary plaques screened, 20 positives for DHR3 and 220 for EcR were detected. Twenty cDNAs for each gene were purified, of which the ten largest for each were restriction mapped and found to be colinear. cDNA DHR3-9, which extends further in both the 5' and 3' directions than our other DHR3 cDNAs, was chosen for sequencing. For EcR, the longest cDNA, EcR-17, extended the farthest 5' and was sequenced in its entirety. An additional cDNA clone, EcR-9, was found to extend 300 bp farther 3' than EcR-17, and this 3' extension was also

sequenced. Additional genomic DNA clones covering the EcR and DHR3 genes wer obtained by screening the Drosophila Canton S genomic library referred to in part A above, either with probes from the respective cDNA clones, or, for overlapping clones, by the chromosomal walk method described in Experimental Example I.

DNA sequence analysis

cDNAs were subcloned into BlueScript vectors (Stratagene), and clones for sequencing were generated by exonuclease III digestion (Henikoff, S., 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359).

Double-stranded plasmids were denatured (Gatermann, K. B., G. H. Rosenberg, and N. F. Kaufer, 1988. Double-stranded sequencing, using mini-prep plasmids, in 11 hours. BioTechniques 6:951-952) and sequenced by the dideoxy chain terminating method (Sanger, F., S. Nicklen, and A. R. Coulson, 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467), using the enzyme Sequenase (U.S. Biochemical). cDNA EcR-17 was completely sequenced on both strands, as was the EcR-9 3' extension. cDNA DHR3-9 was sequenced on both strands for the 5' most 2338 bp, which contains the entire ORF, and the remainder of the long 3' untranslated region was sequenced on one strand.

The exon/intron boundaries in genomic DNA clones were first mapped at low resolution by Southern blot analysis of their restriction fragments probed with labeled cDNAs. Genomic DNA surrounding each exon/intron boundary was subcloned and the nucleotide sequence of these subclones determined as above.

Genomic exons were either sequenced entirely, or for the longer exons, were digested and electrophoresed in parallel with cDNA clones to confirm the colinearity of the genomic and cDNA clones. Shorter exons were

completely sequenced from genomic clones. Longer exons had their boundaries sequenced from genomic clones, and were confirmed to be colinear with the cDNA clones by parallel digestion and electrophoresis of the cDNA and genomic clones.

C. The Predicted Amino Acid Sequence of the EcR and DHR3 Proteins and their Implications

Comparison of the predicted EcR and DHR3 protein sequences to the sequence database and to individual members of the steroid receptor superfamily shows that these proteins share the two conserved domains characteristic of this superfamily (Evans, R. M., 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889-895; Green, S., and P. Chambon, 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends in Genetics 4:309-314). We refer to the domains as the C and E regions, for the more amino-terminal and more carboxy-terminal homologies, respectively, according to the nomenclature of Krust et al. (Krust, A., S. Green, P. Argos, V. Kumar, P. Walter, J. M. Bornert, and P. Chambon, 1986. The chicken oestrogen receptor sequence; homology with v-erbA and the human oestrogen and glucocorticoid receptors. EMBO J. 5:891-897). These domains are underlined in Tables 2 and 3, and Table 4A-C presents a comparison of these domains from EcR and DHR3 with those from representative members of the superfamily.

Table 4. Sequence comparison of the conserved C and E regions in DHR3, EcR, and some representative nuclear receptor homologues. (A) C-region alignment. Numbers at the left indicate the amino acid positions within the individual receptors; dashes indicate gaps introduced to obtain maximal alignment. Dots indicate three positions important in determining the DNA binding specificity of this domain. (B) E-region alignment. Bars indicate the three most highly conserved stretches within this domain. (C) Computed percent identities among the C-region sequences (lower left) and among the E-region sequences (upper right). The kni sequence shows no significant E-region homology and is, therefore, not included in this comparison. Sequences shown are from: E75A, Drosophila ecdysone-inducible gene at 75B; kni, Drosophila segmentation gene knirps; hRARα, human retinoic acid receptor alpha; htRß, human thyroid receptor beta; hVDR, human vitamin D receptor; cOUP-TF, chicken ovalbumin upstream promoter transcription factor; hERR1 and hERR2, human estrogen-related receptors 1 and 2; hER, human estrogen receptor; hGR, human glucocorticoid receptor; hMR, human mineralocorticoid receptor; hGR, human progesterone receptor.

Panel 1 is shown in detail in panels 2-6.

The C region is a 66-68 amino acid domain that has been shown to function as a zinc finger DNA binding domain in vertebrate receptors. This domain has also been implicated in receptor dimerization (Kumar, V., and P. Chambon, 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145-156). As shown in Table 4A, all 19 C-region residues that are absolutely conserved in the other receptor homologues are also conserved in DHR3 and EcR, including the nine invariant Cys residues, eight of which coordinate two zinc ions (Freedman, L. P., B. F. Luisi, Z. R. Korszun, R. Basavappa, P. B. Sigler, and K. R. Yamamoto, 1988. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature 334:543-546). As seen in Table 4C, the Drosophila C-region sequences (including those of E75A) are not more closely related to each other than they are to those from the vertebrate receptor homologues. The C region of DHR3 is most similar to that of the human retinoic acid receptor α (hRARα), and the C region of EcR is most similar to that of the human thyroid receptor ß (hTRß). Studies on the human glucocorticoid receptor (hGR) and human estrogen receptor (hER) have identified three C-region residues (indicated by dots in Table 4A) that are critical for determining the differential DNA binding specificity of these receptors (Mader, S., V. Kumar, H. de Verneuil, and P. Chambon, 1989. Three amino acids of the estrogen receptor are essential to its ability to distinguish an estrogen from a glucocorticoid-responsive element. Nature 338:271-274; Umesono, K., and R. M. Evans, 1989. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 57:1139-46). The three Drosophila proteins DHR3, EcR, and E75A, as well as the vertebrate receptors hRARα, hTRß, and the human vitamin D receptor (hVDR), all have identical amino acids at these three positions; thus, these proteins may

all have similar DNA binding specificities, as has already been shown for hRARα and hTRß (Umesono, K., V. Giguere, C. K. Glass, M. G. Rosenfeld, and R. M. Evans, 1988. Retinoic acid and thyroid hormone induce gene expression through a common responsive element. Nature 336:262-265).

The E-region is an ~225 amino acid domain that functions as a hormone-binding domain in vertebrate receptors. This domain has also been implicated in hormone dependent receptor dimerization (Kumar, V. and P. Chambon, 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145-156; Guiochon, M. A., H. Loosfelt, P. Lescop, S. Sar, M. Atger, A. M. Perrot, and E. Milgrom, 1989. Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. Cell 57:1147-1154), hormone dependent nuclear localization of the glucocorticoid receptor (Picard, D., and K. R. Yamamoto, 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J. 6:3333-3340), and binding of the glucocorticoid receptor to the 90 kDa heat shock protein (Pratt, W. B., D. J. Jolly, D. V. Pratt, W. M. Hollenberg, V. Giguere, F. M. Cadepond, G. G. Schweizer, M. G. Catelli, R. M. Evans, and E. E. Baulieu, 1988. A region in the steroid binding domain determines formation of the non-DNA-binding, 9 S glucocorticoid receptor complex. J. Biol. Chem. 263:267-273). Table 4B shows an alignment of the E regions of the DHR3 and EcR proteins with those of other receptor homologues. The three relatively highly conserved stretches within this region noted in Experimental Example I are overlined; each contains a cluster of residues conserved in all or most of the receptor sequences. DHR3 and EcR show strong similarity to each other and to the other proteins in these stretches, and a lower similarity outside of them. The presence of this

E-region homology establishes these proteins as bona fide members of the nuclear receptor family, in contrast to the Drosophila knirps (Nauber, U., M. J. Pankratz, A. Kienlin, E. Seifert, U. Klemm, and H. Jackle, 1988. Abdominal segmentation of the Drosophila embryo requires a hormone receptor-like protein encoded by the gap gene knirps. Nature 336:489-492), knirps-related (Oro, A. E., E. S. Ong, J. S. Margolis, J. W. Posakony, M. McKeown, and R. M. Evans, 1988. The Drosophila gene knirps-related is a member of the steroid-receptor gene superfamily. Nature 336:493-496), and egon (Rothe, M., U. Nauber, and H. Jackle, 1989. Three hormone receptor-like Drosophila genes encode an identical DNA-binding finger. EMBO J. 8:3087-3094) proteins, which show C-region homology but no E-region homology. The E region in DHR3 is most similar to that of E75A, and the E region of EcR is most similar to that of hTRß, although the level of these similarities is lower than those found among E regions of many other receptors (Table 4C). Thus, DHR3 and EcR are not especially close homologues of any previously cloned receptors. Comparison of E-region sequences allows division of the nuclear receptors into subfamilies (Petkovich, M., N. J. Brand, A. Krust, and P. Chambon, 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330:444-450), the members of any one subfamily being more related to each other than to those in other subfamilies. The DHR3 and EcR receptors fall into a subfamily with the E75A, E75B, hRARα, hTRß, and hVDR receptors.

D. In Situ Labeling of the EcR and DHR3 Proteins with Antibodies Induced by Proteins Produced in E. coli

To determine the intracellular and tissue distribution of the EcR and DHR3 proteins in Drosophila, affinity-purified polyclonal antibodies directed against those proteins were produced in the following manner. The region of about 120 amino acid residues located

between the conserved DNA-binding and hormone-binding domains of these proteins was used as the immunogen to produce antibodies against each protein. Thus, the coding sequences for amino acids 335-447 of the EcR protein and for amino acids 164-289 of the DHR3 protein (see Tables 2 and 3, respectively) were cloned into the appropriate pATH (Dieckmann, C., and A. Tzagaloff, 1985. J. Biol. Chem. 260:1513-1520) or pUR expression vectors, so as to fuse these coding sequences to those encoding E. coli ß-galactosidase (ßgal) or to E. coli tryptophan E protein (trpE), respectively.

The ßgal fusion proteins were produced in E. coli by the addition of the IPTG inducer to exponential cultures, while the production of trpE fusion proteins were induced by dilution into tryptophan-free media and subsequent addition of indoleacetic acid. For EcR, the trpE fusion protein was used as an immunogen and the ßgal fusion protein was used on immunoblots to test sera for immunoreactivity to the EcR portion of the fusions. For DHR3, the ßgal fusion protein was injected, and sera were checked against the trpE fusion protein.

For immunization the appropriate fusion protein was prepared by electrophoresis on SDS-PAGE gels and visualized by staining in ice-cold 0.25 M KCl, after which the fusion protein band was cut out. Approximately 100 µg of fusion protein in 0.25 ml of gel slice was crushed by passing through successively smaller hypodermic needles, and mixed with 0.25 ml of a sterile saline solution and 0.5 ml of Freund's complete adjuvant. For each immunogen, two New Zealand White rabbits were injected at multiple intramuscular sites, and after one month, boosted at two-week intervals, omitting the Freund's adjuvant. While the ßgal fusion proteins were subject to the above gel electrophoresis without prior purificati n, the trpE fusion proteins were first purified by the following method which takes advantage of their insolubility in vivo.

E. coli from a two-liter culture of induced cells were washed, and the cell pellet was subjected to several freeze/thaw cycles. The cells were resuspended in 18 ml of 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 1.8 ml of 10 mg/ml lysozyme was added. After 15 minutes on ice, the cells were lysed by passing three times through a french pressure cell at 10,000 psi. The insoluble fraction was collected by centrifugation at 27,000 x g for 15 minutes, and washed by resuspension, using a Dounce homogenizer, in ice-cold 50 mM Tris-HCl, 0.5 mM EDTA, 0.3 M NaCl, followed by centrifugation as above. The washing step was repeated, and the final pellet dissolved in 10 ml of 4M urea, 2% (w/v) SDS, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5% (v/v) 2-mercaptoethanol. Material remaining insoluble was centrifuged out and discarded.

The antisera was affinity purified in a two-step procedure by successive passage through "nonspecific" and "specific" affinity columns. In the case of antibodies raised against the trpE fusion proteins, the nonspecific column consisted of resin coupled to the insoluble protein derived from E. coli expressing unmodified trpE protein, and was used to remove antibodies directed against trpE epitopes, as well as against insoluble E. coli protein impurities. The specific column consisted of resin coupled to the EcR-trpE fusion protein (purified as described above) and was used to absorb the desired antibodies directed against the EcR epitopes, antibodies that were subsequently released from the column. In the case of antibodies raised against the ßgal fusion proteins, the same general procedure was used, except that the resin in the nonspecific column was coupled to ß-galactosidase, while that in the specific column was coupled to the DHR3-ßgal fusion protein. Western blot analysis of the appropriate E. coli extracts demonstrated that these affinity-purified antibodies exhibited the desired specificity.

The intracellular distribution of the EcR protein in late third instar salivary glands was examined by in situ labeling of this protein with the anti-EcR antibody. The EcR protein was thereby shown to be highly localized in the nuclei of these glands. Indeed, when the polytene chromosomes in these nuclei were examined by the antibody-labeling method of Zink and Paro (Zinc, B., and R. Paro, 1989. Nature 337:468-471), specific loci within these chromosomes exhibited strong binding of the EcR protein. In particular, the EcR protein was bound to the early puff loci, including those occupied by the E75 and E74 genes. This is the result expected if the ecdysone receptor encoded by the EcR gene is that which induces the transcription of the early genes, as anticipated by the Ashburner model. Another prediction of the Ashburner model is that the ecdysone-receptor complex initially represses the genes responsible for the later puff, so that the transcription of the late genes induced by the early gene proteins is delayed until these proteins accumulate sufficiently to overcome this initial repression. If the EcR receptor is involved in this postulated initial repression, then one would expect the EcR protein to bind to the late puff loci in the salivary glands. This expectation was met by the observation that EcR protein also binds to the late puff loci in the polytene chromosomes.

Additional in situ antibody labeling experiments demonstrated that the EcR protein is present in the nuclei of all ecdysone target tissues examined in late third instar larvae. It is also present in most, if not all, cells during embryogenesis and other stages of Drosophila development that have been examined. In this respect, the EcR protein was not detected by anti-EcR antibody labeling of embryos in which the EcR gene was eliminated by a chromosomal del tion, further d monstrating the specificity of this antibody.

In contrast to the widespread distribution of the EcR protein, anti-DHR3 antibody labeling of embryos demonstrated that the distribution of the DHR3 protein is highly restricted during this stage of development. During the brief embryonic period of expression, the protein is restricted to the peripheral nervous system, and to cells surrounding the spiracles at the posterior end of the embryo.

Finally, it should be noted that affinity-purified antibodies against the E75A protein have also been prepared by the same technique described above for anti-EcR and anti-DHR3 antibodies. In situ antibody labeling of the E75A protein in larval salivary glands has also demonstrated that this protein is localized in the nucleus and is bound to specific loci in the polytene chromosomes.

EXAMPLE III

THE ECDYSTEROID-BINDING, DNA-BINDING AND GENETIC REGULATORY PROPERTIES OF THE EcR PROTEIN DEMONSTRATE THAT IT IS AN ECDYSONE RECEPTOR.

The following experiments demonstrate that the protein encoded by the EcR gene is an ecdysone receptor by the following three criteria. (1) The EcR protein binds ecdysteroids and accounts for a large proportion, if not all, of the ecdysteroid-binding activity present in Drosophila embryos and in a variety of cultured Drosophila cells. (2) The EcR protein binds with high specificity to a DNA sequence that functions as an ecdysone response element (EcRE), i.e., an enhancer that confers ecdysone inducibility to a promoter. (3) Cells that do not respond to ecdysone because they lack functional ecdysone receptors are transformed to the ecdysone-responsive state by transfection with an EcR expression plasmid.

A. The EcR Protein Binds Ecdysteroids

The EcR xpression plasmid, pMTEcR, shown in Figur 1 contains the open reading frame encoding the EcR protein (EcR ORF; see Experimental Example II) fused to the Drosophila metallothionine promoter (P_{MT}) at its 5' end, and the polyadenylation-cleavage sequences of the Drosophila Actin 5C gene at its 3' end. Because transcription of the EcR ORF is under the control of this metallothionine, that transcription is induced by Cu^{2+} ion to yield an mRNA that, in turn, produces the EcR protein. A cell line, MtEcRHy, that overproduces this protein upon Cu^{2+} induction, as determined by Western blot analysis using the affinity-purified anti-EcR antibody (see Experimental Example II), was constructed by the stable integration of the pMTEcR plasmid DNA into the genome of Drosophila Sch-2 cell line. A control cell line, MtHy, was similarly constructed by the integration of the expression vector DNA lacking the EcR ORF.

Whole cell extracts were prepared from both the MtEcRHy and MtHy cell lines after Cu^{2+} induction, and were assayed for ecdysteroid-binding activity using the high affinity ecdysone analogue [^{125}I] iodoponasterone A. The MtEcRHy extract contained sevenfold more saturable ecdysteroid-binding activity than the MtHy control extract.

To see if the induced ecdysteroid-binding activity was due to the EcR polypeptide itself, the EcR protein was depleted from the MtEcRHy extract by immunoprecipitation using an affinity-purified anti-EcR polyclonal antibody, or, as a control, the extract was mock-depleted with preimmune serum. The treated extracts were then assayed for ecdysteroid-binding activity. Comparison of the immuno-depleted extract with the mock-depleted extract showed that most of the binding activity was removed by the anti-EcR antibody treatment, indicating that the induc d ecdysteroid-binding activity results from the EcR protein.

The endogenous ecdysteroid-binding activity in the control cell line, MtHy, was unchanged by Cu^{2+} exposure, and was approximately the same as that in the Sch-2 cell from which it derives. The question arises as to whether the endogenous activity in these and other Drosophila cell lines, as well as in embryonic extracts, results from the expression of the EcR gene in their respective genomes. To answer this question, extracts from embryos and several cell lines were immuno-depleted and mock-depleted, as described above, and assayed for ecdysteroid-binding activity. Again, comparison of these treated extracts showed that the large majority of the endogenous binding activity was removed in each case by treatment with the anti-EcR antibody. Thus, it appears that most, if not all, of the endogenous binding activity in embryos and cell lines results form the resident EcR gene.

Methods

Extracts

Tissue culture cell extracts for hormone and DNA-binding experiments were prepared as follows. Cells were grown in spinner flasks to a density of $5-7x10^6$ cells/ml, and were washed once in EcR buffer (25 mM Hepes, pH 7.0, 40 mM KCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, and the following cocktail of protease inhibitors: 10 mM $Na_2S_2O_5$, 500 μM PMSF, 1 μM leupeptin, 1 μM pepstatin). All further manipulations were at 4°C. Cells were resuspended in EcR buffer at 2% of the original culture volume, divided into 3 ml aliquots, and sonicated using 30 1/2 second pulses with a probe sonicator (Bronson Sonifier 450), resulting in disruptions of ~95% of the cells. After centrifugation at 100,000 x g for 1 hour, 100 μl aliquots of supernatant were frozen in liquid nitrogen, and stored at -80°C. Protein concentration was determined using bovine serum albumin as the standard, and was typically 6-11 mg/ml.

Embryo extracts were prepared by a similar protocol: 3-6 hour Canton S embryos were d chorionated in 55% commercial bleach for 2 minutes, washed extensively in 0.7% NaCl, and resuspended using 2 grams of embryos per ml of EcR buffer. Embryos were broken with 20 strokes in a Dounce homogenizer using a B pestle, and lysis was completed with the probe sonicator using the same settings as used for the tissue culture cells. The extract was adjusted to 400 mM KCl, centrifuged 1 hour at 100,000 x g, and aliquots of supernatant were frozen. This extract contained 13.4 mg/ml protein. Before use in hormone binding, it was diluted tenfold in EcR buffer lacking KCl to bring the final KCl concentration to 40 mM.

Hormone-binding assays

For hormone-binding experiments, extracts were first diluted to the following concentrations in EcR buffer: 0.9 mg/ml for MtHy and MtEcRHy extracts, 3 mg/ml for S2 and SRS 1.5 extracts, 4 mg/ml for the Kc cell extracts, and 1.3 mg/ml for the embryo extract. All manipulations were done on duplicate samples in order to quantify variability in the results. For immunoprecipitation experiments, extracts were immuno-depleted, mock-depleted, or left untreated. For depletions, 300 µl of diluted extract was incubated for 30 minutes at 25°C with 3.5 µl affinity-purified anti-EcR antibody, or with 3.5 µl preimmune serum for the mock-depletion control. Then 38 µl 10% Staphylococcus aureus (Pansorbin, Calbiochem) in EcR buffer was added, and incubation was continued for 15 minutes at 25°C. After centrifugation for 3 minutes in a microcentrifuge, the supernatant (depleted extract) was recovered. The immunoprecipitation was repeated, except in the case of the embryo extract, which was subjected to only one round of precipitation. The "untreated" extract aliquots were left at 4°C for the duration of the depletion procedure,

and were diluted with EcR buffer to match the final concentration of the depleted aliquots.

A modification of the hormone-binding assay of P. Cherbas was used (Cherbas, P. 1988. Proc. Nat'l Acad. Sci., U.S.A. 85:2096-2100). Assay tubes contained 140 µl extract, 14 µl [^{125}I] iodoponasterone, and either 14 µl EcR buffer or 14 µl unlabelled 20-OH ecdysone in EcR buffer as a competitor. [^{125}I] iodoponasterone was 2177 Ci/mM and was used at a final concentration of $5x10^{-10}$ M in the assay; 20-OH ecdysone was $2x10^{-5}$ M final concentration in the assay. After incubation for 1 hour at 25°C, each reaction was spotted on a dry Whatman GF/C filter (2.4 cm), and after 30 seconds the filter was washed by using a vacuum to draw 10 ml EcR buffer through the filter over a period of 1 minute. Filters were placed in 800 µl 4% SDS, and radioactivity was measured in a γ counter. The hormone-binding activities shown are saturable binding activities, calculated as the total binding activity, as measured in assays with no added competitor, minus the unsaturable binding activity, which was measured in the assays with excess unlabelled ecdysone added. In the most active extracts, the unsaturable activity (representing the large number of low affinity binding sites in the extract) was less than 10% of the total activity.

B. Genetic Regulatory Activity of the EcR Protein in vivo

An ecdysone-inducible reporter plasmid, pEcRE/Adh/ßgal (Figure 2), was constructed to test the regulatory functions of the EcR protein in vivo. The reporter gene in this plasmid consists of the sequence that encodes the E. coli ß-galactosidase (ßgal ORF) linked through the 5' leader sequence of the Drosophila Ultrabithorax gene (UBX leader and AUG) to an ecdysone-inducible promoter. This promoter was created by fusing a truncated version of the proximal promoter for the

Drosophila Adh gene ($P_{DAdh-34+53}$, the numbers indicating that it consists of the sequence from base pair positions -34 to +53, which just includes the TATA box) to seven repeats of a 34 bp synthetic oligonucleotide (7 EcRE OLIGOS) which contains the ecdysone response element (EcRE) from the ecdysone-inducible heat shock gene hsp 27 (Riddihough and Pelham, 1987. EMBO J. 6:3729-3734). The seven EcREs should confer ecdysone-inducibility to the truncated promoter, provided that the cells transfected with this reporter plasmid contain the appropriate ecdysone receptor.

This ecdysone-inducible reporter plasmid was constructed by insertion of the 7 EcRE OLIGOS into plasmid pAdh/βgal, which is identical to pEcRE/Adh/βgal except that it lacks the array of ecdysone response elements. The pAdh/βgal plasmid should therefore not be ecdysone inducible and can serve as a control. To test these expectations, Sch-2 cultured cells (which were shown above to contain endogenous ecdysone-binding activity) were transfected with each plasmid and examined for β-galactosidase activity in the presence and absence of ecdysone. The ecdysone-induced β-galactosidase activity in the pEcRE/Adh/βgal transfected cells was 2000-fold greater than when such cells were not exposed to ecdysone, whereas ecdysone had little effect on the pAdh/βgal transfected cells. These results indicate that the EcREs confer ecdysone-inducibility on the $P_{DAdh-34+53}$ promoter, as expected, and that the Sch-2 cells contain functional ecdysone receptors.

To test the function of the EcR receptor in such a system, host cells lacking functional ecdysone receptors are required. "Ecdysone-resistant" cells lacking ecdysone-binding activity, and hence, presumably functional receptors, can be produced by continuously exposing ecdysone-responsive cells to ecdysone during a period of several weeks. This ecdysone-resistant state is then maintained in ecdysone-free media for several

months. An ecdysone-resistant cell line, SRS 1.5, was therefore generated by growing Sch-2 cells in $3x10^{-6}$ M ecdysone. The SRS 1.5 cells lack significant ecdysone-binding activity.

When these cells were transfected with the pEcRE/Adh/ßgal plasmid and subsequently exposed to ecdysone, very little ecdysone-induced ß-galactosidase activity was observed, indicating that the cells have only trace amounts, if any, of functional receptors. To test whether the expression of the EcR gene can "rescue" this deficiency, the SRS 1.5 cells were cotransfected with two plasmids: the ecdysone-inducible reporter plasmid, PEcRE/Adh/ßgal, and a constitutive expression plasmid for the EcR gene, pActEcR, in which transcription of the EcR ORF is controlled by the Drosophila Actin 5C promoter, P_{Act5C} (Figure 3). Cotransfection with these two plasmids, followed by exposure to ecdysone, resulted in a dramatic induction of ß-galactosidase activity. Thus, introduction of this EcR expression plasmid into the SRS 1.5 cells regenerated the ecdysone-inducibility they had lost.

Methods

Construction of the pAdh/ßgal, pEcRE/Adh/ßgal and pActEcR plasmids

Plasmid pAdh/ßgal was constructed in two steps. The BglII-ScaI fragment of pDΔ5'-34, containing nucleotides -34 to +53 of the Drosophila Adh distal promoter, was cloned into pUC18 cut with ScaI and BamHI. The resulting plasmid was cut with EcoR1, and the EcoR1 fragment of cPßbxd6.2 (containing the Ubx untranslated leader and AUG, the ßgal open reading frame, and the SV40 splice and poly A signals) inserted.

To construct pEcRE/Adh/ßgal from pAdh/ßgal, two 34-residue oligonucleotides were synthesized:

5'TCGAGAGACAAGGGTTCAATGCACTTGTCCAATG3'
3'CTCTGTTCCCAAGTTACGTGAACAGGTTACAGCT5'

These will anneal to form 30 bp duplexes with Sal1 compatible four nucleotid overhangs at their 5' ends, as shown. Further annealing via the 5' overhangs allows formation of tandem arrays that can be inserted into pAdh/Bgal at its Sal1 site just upstream from the TATA box of the truncated Adh promoter. When these oligonucleotides were kinased, annealed, ligated into Sal1-cut pAdh/Bgal and cloned, pEcRE/Adh/Bgal was obtained. Restriction mapping showed that it contained a tandem array of seven 34 bp repeats, each of which contains the 23 bp ecdysone response element (EcRE) present in the hsp 27 gene, the remaining 11 bp representing flanking hsp 27 sequences and the 5' overhangs.

The constitutive EcR expression plasmid, pActEcR, was formed by inserting the Fsp1-HpaI fragment of an EcR cDNA containing bp 851-4123 that contains the ORF encoding the EcR protein (Table 2), into the EcoRV site of the ActSV40BS plasmid. This expression vector was constructed in two steps by inserting the Xba1-EcoR1 fragment of cosPneoß-gal, containing the SV40 splice and poly A signals, into BlueScript+KS (Stratagene) cut with SacII and Xba1, blunting the EcoR1 and SacII ends. The resulting plasmid was digested with BamH1 and Apa1, and the BamH1-EcoR1 fragment of pPAc was inserted, with the Apa1 and EcoR1 ends being blunted.

Transfection and generation of the cell line SRS 1.5

The cell line SRS 1.5 was obtained by growing Schneider line 2 (Sch-2) cells in the presence of $3x10^{-6}$ M 20-OH ecdysone (Sigma). This treatment initially halts growth of Sch-2 cells, but after several weeks the adapted cells grow well. SRS 1.5 cells were washed in hormone-free medium and passed several times in hormone-free medium prior to their use in transfection experiments. Cells wer transfected by the calcium phosphate technique. Cells were transfected with 10 μg

of each plasmid used; when only a single plasmid was being transfected, 10 μg of pUC18 DNA was added as a carrier. In general, all transfections were carried out in duplicate. Twenty-four hours after transfection, cells that were to undergo hormone treatment were split into two dishes, one of which was treated with $2x10^{-6}$ M 20-OH ecdysone.

ß-galactosidase assays

Forty-eight hours after transfection, 2 ml of cells were washed once in PBS (137 mM NaCl, 27 mM KCl, 65 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 6.8), and were resuspended in 50 μl of 0.25 M sucrose, 10 mM Tris, pH 7.4, 10 mM EDTA, and repeatedly frozen in liquid nitrogen and thawed in a 37°C water bath for a total of 3 freeze/thaw cycles. Cell debris was removed by a 10-minute centrifugation in a microcentrifuge at 4°C. The concentration of protein in the supernatant (cell extract) was determined by the Bradford method, with bovine serum albumin as a standard, and was typically 1.5-2.5 mg/ml. Extracts were assayed immediately or frozen and assayed up to two weeks later with no loss in activity. To 10 μl of extract, or an appropriate dilution, 500 μl of assay buffer was added (0.6 mM 4-methylumbelliferyl-ß-D-galactoside, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.0 mM $MgSO_4$, pH 7.0). After a 30-minute incubation at 37°C, reactions were stopped with 500 μl of 300 mM glycine, 15 mM EDTA, pH 11.2. The fluorescent reaction product was quantified on a Perkin-Elmer LS-5B luminescence spectrometer, with λ_{ex}=365 nm and λ_{em}=450 nm. ßgal activities are given as fluorescence units per μg protein assayed.

C. Specific Binding of the EcR Protein to Ecdysone Response Elements

The simplest explanation of the results described in the preceding section is that the EcR protein generated by the EcR expression plasmid binds to the EcRE of the

reporter plasmid and, in combination with ecdysone, activates th minimal Adh promoter in that plasmid. The following experiment was designed to test whether the EcR protein exhibits specific binding to this EcRE in vitro.

Two plasmids were used: pUC18, which serves as the control, and pUC18-EcRE, which was generated by substituting the HindII-XbaI fragment from pEcRE/Adh/βgal that contains the seven repeats of the 34 bp EcRE oligonucleotide, for the HindII-XbaI fragment of pUC18. Because the only difference between these two fragments is the seven oligonucleotide repeats, this is also the only difference between the two plasmids.

The two plasmids were digested with ApaLI and Hind III, end-labeled with ^{32}P and mixed with an extract from MtEcRHy cells in which the EcR protein was overexpressed by Cu^{2+} induction (see section A, above). After a 15-minute incubation at 25°C to allow EcR-DNA binding to occur, affinity-purified anti-EcR antibody was added. The 25°C incubation was continued for an additional 40 minutes, at which time anti-rabbit Ig-coated magnetic beads (Dupont Magnasort-R) were added, and the incubation continued 15 minutes more. The beads were separated from the solution magnetically, similarly washed, and the DNA eluted from the beads in 1% SDS at 65°C. The eluted DNA was ethanol precipitated and fractionated by electrophoresis in an agarose gel, which was dried and autoradiographed.

Only the fragment containing the EcRE oligonucleotide was specifically and efficiently registered on the autoradiographs, and that registration was dependent upon the anti-EcR antibody. Quantitative analysis of the autoradiographs demonstrated a 10^3-fold preference for binding to the EcRE oligonucleotide over the average vector sequences, under the conditions of this assay (see Methods, bel w).

According to the criteria stated at th beginning of this Experimental Example, the EcR protein clearly satisfies the definition of an ecdysone receptor.

Methods

Conditions for the DNA binding assay

A quantity of 0.2 fmole of digested, labelled plasmid DNA was mixed with 2 μg (dI/dC) in 10 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and 90 μl of the MtEcRHy extract, diluted to 0.9 mg/ml in EcR buffer adjusted to 180 mM KCl, was added. After binding for 15 minutes at 25°C, 2 ml of affinity-purified anti-EcR antibody, diluted 1.5x in EcR, was added, and this incubation was continued at 25°C for 40 minutes, when 50 μl of anti-rabbit Ig-coated magnetic beads (Dupont Magnasort-R), exchanged into 180 mM KCl EcR buffer, was added and the incubation continued for 15 minutes.

The beads were washed twice in 400 μl 180 mM KCl EcR buffer, and DNA was eluted from the beads by soaking twice in 200 μl 1% SDS in TE at 65°C. The eluted DNA was ethanol precipitated and run on an agarose gel, which was dried and autoradiographed. As controls, one half of the input DNA (0.1 fmole) was run on the gel for comparison, and the binding assay was carried out, leaving out the antibody.

EXAMPLE IV

RECEPTOR GENE MUTAGENESIS.

Mutations in the steroid receptor superfamily genes can alter their function in two ways. Most obviously, they alter the sequences encoding the receptor proteins and thus alter the receptor function. Alternatively, they can alter the expression of these genes -- an alteration that can be at any level of that expression from transcription of the gene to the translation of its mRNA(s). Such mutations can change the timing of gene expression during development or change the tissue and

cell distribution of that expression, thus, profoundly changing the course of development. Furthermore, these mutations provide information about the regulation of receptor gene expression, just as mutations that alter the structure of the receptors encoded by these genes provide information about the genes whose expression these receptor proteins control. In particular, mutations that alter receptor gene expression can lead to the identification of the proteins and other regulatory molecules that control their expression. Clearly, mutagenesis of insect steroid receptor superfamily genes provides an important avenue leading to an ability to interfere in a highly specific manner with insect development, and thus to control insect infestations deleterious to human health and agriculture.

We have carried out mutagenesis experiments for two Drosophila members of the steroid receptor superfamily genes, E75 and EcR, that we have cloned and characterized with respect to their expression. In this experimental example, mutagenesis of the E75 gene is described.

A. Deletion Mutations

In Drosophila, genetic analysis for a given locus -- in this case, the early puff locus at 75B that houses the E75 gene -- generally depends upon the isolation of deletions of all or part of that locus. Such deletions greatly facilitate the subsequent isolation of point and other small mutations within the locus. By isolating mutations that are revertants to the neighboring dominant Wrinkled (W) mutations, we have isolated and molecularly mapped the boundaries within our chromosomal walk (see Experimental Example I) of two deletions, W^{R4} and W^{R10}, generated by gamma ray mutagenesis, the preferred way of generating such large alterations of genomic structure. One of these, W^{R10}, extends distally from Wrinkled to cover the entire E75 g ne; and the other, W^{R4}, extends to a point about 90 kb upstream of the 5' end of the 50 kb

E75A transcription unit and does not include the E75 gene.

An F2 screen was then employed to screen for gamma ray-induced mutations mapping to the 200 kb distal region that is included in the W^{R10} deletion but not the W^{R4} deletion. This screen resulted in the isolation of five members of a single lethal complementation group that molecular mapping data demonstrate represents the E75 gene. The most useful of these five mutations is the $E75^{X48}$ mutation. Molecular mapping of this mutation demonstrated that it is a 105 kb region that includes all of the E75 gene. This method provides an extremely efficient screen for other E75 mutations, i.e., by screening for mutations that cannot complement this deletion mutation.

B. E75 Mutations Generated by Ethyl Methane Sulfonate

The chemical mutagen ethyl methane sulfonate (EMS) was used for this screen, as it is the preferred method for generating point or small mutations. An F2 screen of 15,000 lines resulted in the isolation of 23 penetrant mutations within the 105 kb region of the $E75^{X48}$ deletion, all of which turned out to be alleles of E75. It appears that this 105 kb region was saturated by this screen with respect to lethal complementation groups, and hence, E75 appears to be the only lethal complementation group in this region. Adding the five E75 mutations described above, a total of 28 penetrant E75 alleles have thus been isolated, several of which are temperature-sensitive alleles.

Inter se complementation studies among these alleles and examination of their phenotypes reveal a complex complementation group -- a complexity that probably results from the fact that the E75 gene contains two overlapping transcription units, a 50 kb E75A unit and a 20 kb E75B unit that occupies the 3' end of the E75A unit

(see Experimental Example I and Table 1). These alleles can be roughly divid d into two groups: (1) those that cause lethality in early development, during the latter part of embryogenesis or during early larval development, and (2) those that cause lethality late in development, during the prepupal or pupal stages.

This division correlates with the stages when the E75A and E75B units are expressed. Thus, E75A transcription is associated with each of the six pulses of ecdysone, including those that mark the embryonic and early larval stages. By contrast, E75B mRNAs are not observed until the end of the last larval stage, being particularly abundant during the pupal stage. This correlation invites the speculation that the early lethal mutations affect the expression of the E75A unit and its E74A protein, and that the late lethal mutations specifically affect the expression of the E75B unit and its E75B protein. This proposition can be tested by detailed molecular mapping of these mutations and further examination of their phenotypes at the molecular level to determine the causes of lethality.

The mutants described here provide a foundation for the further genetic analysis of the E75 gene that will allow exploration of the requirements for appropriate E75 expression and function and will identify structural and functional domains of E75. Some of the future E75 studies will best be performed by its in vitro manipulation, followed by transformation of the constructs back into Drosophila. Finally, it will be desirable to identify interacting genetic loci -- interactions that may occur at the level of regulation of E75 expression or at the level of interaction of the E75 proteins with those encoded by other genes. Such interactive genetic loci can be identified via the isolation of mutations that act as suppressors or enhancers of the E75 mutations.

Methods

Strains, markers and chromosomes

For this aspect of the invention, the following strains, markers and chromosomes were used. Tu^2 was described by Lindsley (Lindsley, 1973. DIS 50:21). All other strains and mutations are as described (Lindsley, and Grell, 1968. Genetic Variation of *Drosophila melanogaster*, Publication 627, Carnegie Institute of Washington, Washington, DC). ru h W^{R4} e^s ro ca was constructed by recombination between ru h W^{R4} sbd^2 Tu^2 and st sbd^2 e^s ro ca. The st in ri p^p sbd^2 chromosome was constructed by recombination of st in ri p^p with sbd^2, in order to allow marking of this chromosome over W^{R4} and W^{R10}, and homozygosed by crossing to TM3, backcrossing to TM3, and mating of isogeneic sibling progeny. The st p^p e11 line was homozygosed by standard ionic procedures. $Antp^W$ and ns^{Rc4} are described in Scott et al. (1984) Proc. Nat'l Acad. Sci. USA 81:4115-4119. The pupal lethals X19, g26, Q13B, 8m12, iX-14, 2612, m45, p4, g30L, mz416, 13m115, O52 and wg49 are described in Shearn (1974) Genetics 77:115-125. All strains used to construct the strains described above and other strains were obtained from the Bowling Green and Caltech stock centers.

TM1, TM3 and TM6B (Lindsley, and Grell, 1968. Genetic Variation of Drosophila melanogaster, Publication 627, Carnegie Institute of Washington, Washington, DC) are balancer chromosomes carrying recessive lethal mutations along with multiple inversions to suppress recombination. This allows the maintenance, as a heterozygote, of a recessive lethal chromosome in its original state. These chromosomes are also marked with convenient visible markers.

Quantitative Southern blot mapping for detection of mutant lesions

DNA was prepared from adult flies (about 50) by douncing in 1 ml of 10 mM Tris-HCl, pH 7.5, 60 mM NaCl, 10 mM EDTA, 0.15 mM spermine, 0.2 mg/ml proteinase K. The homogenate was added to an equal volume of 0.2 M Tris-HCl, pH 9.0, 30 mM EDTA, 2% SDS, 0.2 mg/ml proteinase K, incubated at 37°C for 1 hour, and then extracted twice with buffer-saturated phenol and once with 24:1 chloroform/isoamyl alcohol. DNA was EtOH precipitated twice, hooking the pellet out without centrifugation. Southern blot hybridization was as described (Segraves, W. et al., 1984. J. Mol. Biol. 175:1-17). Where restriction fragment length polymorphism was not used in order to distinguish the parental chromosome from the balancer chromosome, quantitation of band intensity on genomic Southerns was achieved using a scanning densitometer. By using a control probe outside the mutant region, the amount of DNA in each track was internally controlled. Comparison of deficiency heterozygote to wild type bands, when normalized to a control band in this way, gives little deviation from the expected 1:2 ratio.

Molecular cloning of mutant lesions

Restriction fragments of the appropriate size were isolated by preparative low melting agarose (FMC) electrophoresis of about 20 μg of restricted genomic DNA. The 6 kb $\underline{W}^{R4}$ XhoI fragment was cloned into XhoI-cleaved λSE6DBam which is propagated as a plasmid in order to grow the vector and cannot be packaged without an insert. The 18 kb $\underline{W}^{R10}$ SalI fragment was cloned into the SalI site of λEMBL3, cleaved also with EcoRI for the biochemical selection method for the prevention of propagation of non-recombinant clones. The 7 kb EcoRI fragment containing the x37 breakpoint was cloned into EcoRI-cleaved λ607. Plating of recombinants on the hflA strain RY1073 prevent d plaque formation by non-recombinant phage. The 14 kb x48 EcoRI fragment was cloned into the

EcoRI site of λEMBL4, which had been cleaved with BamHI to utilize the "biochemical selection" for recombinants. The breakpoint fragments of x44 and the recipient fragment were cloned into λSE6ΔBam. Libraries were packaged using λ in vitro packaging extracts prepared as described in Hohn (Hohn, B., 1979. Methods Enzymol. 68:299-303). After demonstration that each of the libraries gave a significant number of plaques only when inserts were included in the ligation, they were screened using restriction fragments capable of detecting the breakpoint clones.

Gamma ray mutagenesis

Adult males of the strain ru h W sbd^2 Tu^2 or st in ri p^p sbd^2 were irradiated in plastic vials with 5000 rad of gamma rays from a Cs^{137} source at a dose rate of 4300 rad/minute. These were then mated to virgins of the appropriate strain, which were allowed to lay eggs for five days.

EMS mutagenesis

The primary lesion in EMS-induced mutations of bacteria and yeast is an alkylation-induced transition of guanine to adenine; most EMS-induced point mutations in Drosophila can similarly be explained on this basis. This change would be expected to convert, on the complementary strand, a C in the opa repeat element to a T, creating an in-frame stop codon (CAGCAA to UAGCAA or CAGUAA). (Ethylnitrosourea, ENU, which has been reported to yield a higher number of mutations for a given amount of sterility, is also an alkylator; however, considerably more stringent precautions must be taken in handling this mutagen.)

EMS was administered at 0.025 M to unstarved 1.5-5 day-old males in 1% sucrose solution (1.5 ml on two slips of Whatman #2 in a 350 ml milk bottle). Starvation of the males for 8 hours before EMS administration resulted

in unacceptable levels of sterility, and males of the st p^p e^{11} strain readily fed upon the EMS/sucrose solution without starvation. Mutagenesis was monitored by crossing mutagenized males to attached-X FMA3 females. Other mutants seen in this screen included a large number of ca alleles (many mosaic) seen over TM6B in the F1 and F2 generations, a dominant brown allele, and two new mutants, Wink, a third chromosome dominant mutation resembling Bar, and a third chromosome dominant Curly-like mutation. Wink is easily scored (RK1), has complete penetrance, and is quite healthy over TM6B.

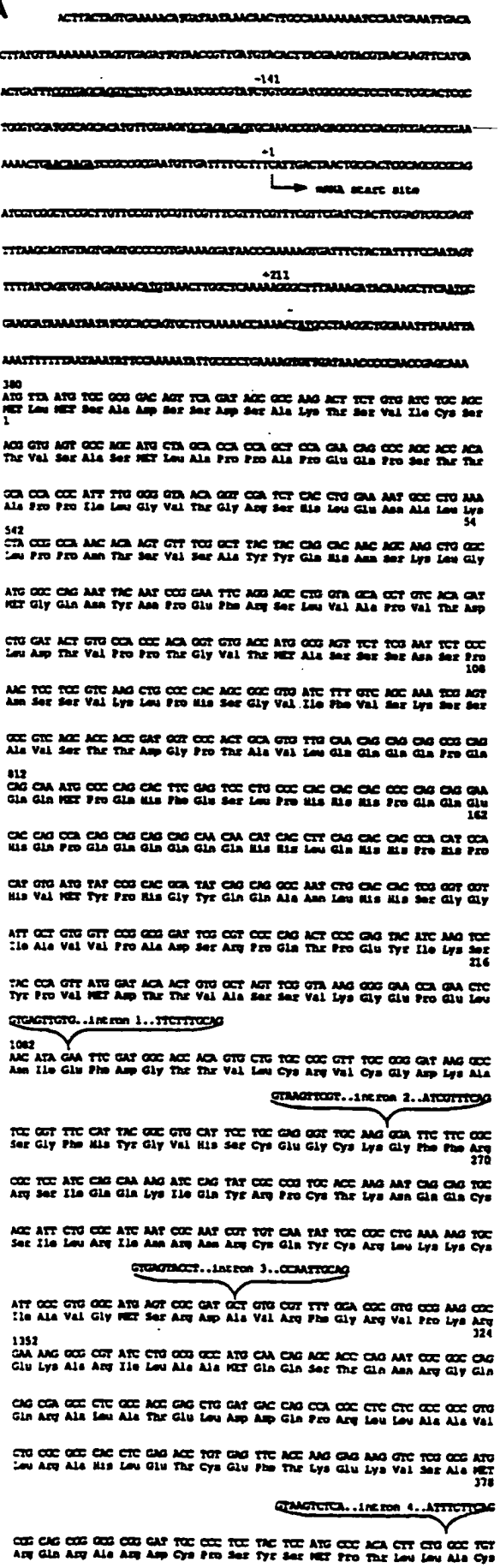
In the initial screen, vials were scored as mutant if they had fewer than 25% as many deficiency heterozygote as balancer heterozygote flies. On retesting, this was revised to 50% of the level seen in control crosses. Balancer heterozygotes were approximately two-thirds as viable as deficiency heterozygotes.

In situ hybridization and cytological analysis

In situ hybridization of polytene chromosomes was carried out as described in Experimental Example I (see Methods, section A). Cytological analysis was performed by squashing larval salivary glands in lactoacetic orcein (2% orcein, 50% acetic acid, 30% lactic acid).

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the claims.

A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-1
mRNA start site
+211
380
ATG TTA ATG TCC GCG GAC AGT TCA GAT AGC GCC AAG ACT TCT GTG ATC TGC AGC
MET Leu MET Ser Ala Asp Ser Ser Asp Ser Ala Lys Thr Ser Val Ile Cys Ser
1
ACG GTG AGT GCC AGC ATG CTA GCA CCA CCA GCT CCA GAA CAG CCC AGC AGC ACA
Thr Val Ser Ala Ser MET Leu Ala Pro Pro Ala Pro Glu Gln Pro Ser Thr Thr
GCA CCA CCC ATT TTG GGG GTA ACA GGT CGA TCT CAC CTG GAA AAT GCC CTG AAA
Ala Pro Pro Ile Leu Gly Val Thr Gly Arg Ser His Leu Glu Asn Ala Leu Lys
54
542
CTA CCC CCA AAC AGA AGT GTT TCG GCT TAC TAC CAG CAC AAC AGC AAG CTG GCC
Leu Pro Pro Asn Thr Ser Val Ser Ala Tyr Tyr Gln His Asn Ser Lys Leu Gly
ATG GCC CAG AAT TAC AAT CCG GAA TTC AGG AGC CTG GTA GCA CCT GTC ACA GAT
MET Gly Gln Asn Tyr Asn Pro Glu Phe Arg Ser Leu Val Ala Pro Val Thr Asp
CTG GAT ACT GTG CCA CCC ACA GGT GTG ACC ATG GCG AGT TCT TCG AAT TCT CCC
Leu Asp Thr Val Pro Pro Thr Gly Val Thr MET Ala Ser Ser Ser Asn Ser Pro
108
AAC TCC TCC GTC AAG CTG CCC CAC AGC GGC GTG ATC TTT GTC AGC AAA TCG AGT
Asn Ser Ser Val Lys Leu Pro His Ser Gly Val Ile Phe Val Ser Lys Ser Ser
GCC GTC AGC ACC ACC GAT GGT CCC ACT GCA GTG TTG CAA CAG CAG CAG CCG CAG
Ala Val Ser Thr Thr Asp Gly Pro Thr Ala Val Leu Gln Gln Gln Gln Pro Gln
812
CAG CAA ATG CCC CAG CAC TTC GAG TCC CTG CCC CAC CAC CAC CCC CAG CAG GAA
Gln Gln MET Pro Gln His Phe Glu Ser Leu Pro His His His Pro Gln Gln Glu
162
CAC CAG CCA CAG CAG CAG CAG CAA CAA CAT CAC CTT CAG CAC CAC CCA CAT CCA
His Gln Pro Gln Gln Gln Gln Gln Gln His His Leu Gln His His Pro His Pro
CAT GTG ATG TAT CCG CAC GGA TAT CAG CAG GCC AAT CTG CAC CAC TCG GGT GGT
His Val MET Tyr Pro His Gly Tyr Gln Gln Ala Asn Leu His His Ser Gly Gly
ATT GCT GTG GTT CCG GCG GAT TCG CGT CCC CAG ACT CCC GAG TAC ATC AAG TCC
Ile Ala Val Val Pro Ala Asp Ser Arg Pro Gln Thr Pro Glu Tyr Ile Lys Ser
216
TAC CCA GTT ATG GAT ACA ACT GTG GCT AGT TCG GTA AAG GGG GAA CCA GAA CTC
Tyr Pro Val MET Asp Thr Thr Val Ala Ser Ser Val Lys Gly Glu Pro Glu Leu
GTGAGTCGTG..intron 1..TTCTTTCCAG
1082
AAC ATA GAA TTC GAT GGC ACC ACA GTG CTG TCC CGC GTT TCC GGG GAT AAG GCC
Asn Ile Glu Phe Asp Gly Thr Thr Val Leu Cys Arg Val Cys Gly Asp Lys Ala
GTAAGTCCGT..intron 2..ATCGTTTCAG
TCC GGT TTC CAT TAC GGC GTG CAT TCC TGC GAG GGT TGC AAG GGA TTC TTC CGC
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270
CGC TCC ATC CAG CAA AAG ATC CAG TAT CGC CGG TGC ACC AAG AAT CAG CAG TGC
Arg Ser Ile Gln Gln Lys Ile Gln Tyr Arg Arg Cys Thr Lys Asn Gln Gln Cys
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Ser Ile Leu Arg Ile Asn Arg Asn Arg Cys Gln Tyr Cys Arg Leu Lys Lys Cys
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ATT GCC GTG GGC ATG AGT CGC GAT GCT GTG CGT TTT GGA CGC GTG CGG AAG CGC
Ile Ala Val Gly MET Ser Arg Asp Ala Val Arg Phe Gly Arg Val Arg Lys Arg
324
1352
GAA AAG GCG CGT ATC CTG GCG GCC ATG CAA CAG AGC ACC CAG AAT CGC GGC CAG
Glu Lys Ala Arg Ile Leu Ala Ala MET Gln Gln Ser Thr Gln Asn Arg Gly Gln
CAG CGA GCC CTC GCC ACC GAG CTG GAT GAC CAG CCA CGC CTC CTC GCC GCC GTG
Gln Arg Ala Leu Ala Thr Glu Leu Asp Asp Gln Pro Arg Leu Leu Ala Ala Val
CTG CGC GCC CAC CTC GAG ACC TGT GAG TTC ACC AAG GAG AAG GTC TCG GCG ATG
Leu Arg Ala His Leu Glu Thr Cys Glu Phe Thr Lys Glu Lys Val Ser Ala MET
378
GTAAGTCTCA..intron 4..ATTTCTTCAG
CGC CAG CGG GCG CGG GAT TCC CCC TCC TAC TCC ATG CCC ACA CTT CTG GCC TCT
Arg Gln Arg Ala Arg Asp Cys Pro Ser Tyr Ser MET Pro Thr Leu Leu Ala Cys
CCG CTG AAC CCC GCC CCT GAA CTG CAA TCG GAG CAG GAG TTC TCG CAG CGT TTC
Pro Leu Asn Pro Ala Pro Glu Leu Gln Ser Glu Gln Glu Phe Ser Gln Arg Phe
1622
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Leu Phe Val Arg Leu Ile Cys MET Phe Asp Ser Ser Ile Asn Ser Ile Ile Cys
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486
TTC CTG GTG GAC TCC ACC TTC AAT TTC GCG GAG CGC ATG AAC TCG ATG AAC CTG
Phe Leu Val Asp Ser Thr Phe Asn Phe Ala Glu Arg MET Asn Ser MET Asn Leu
1892
ACA GAT GCC GAG ATA GGC CTG TTC TCC GCC ATC GTT CTG AAT ACG CGG GAT CGC
Thr Asp Ala Glu Ile Gly Leu Phe Cys Ala Ile Val Leu Ile Thr Pro Asp Arg
CCC GGT TTG CGC AAC CTG GAG CTG ATC GAG AAG ATG TAC TCG CGA CTC AAG GGC
Pro Gly Leu Arg Asn Leu Glu Leu Ile Glu Lys MET Tyr Ser Arg Leu Lys Gly
540
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Cys Leu Gln Tyr Ile Val Ala Gln Asn Arg Pro Asp Gln Pro Glu Phe Leu Ala
AAG TTG CTG GAG ACG ATG CCC GAT CTG CGC ACC CTG AGC ACC CTG CAC ACC GAG
Lys Leu Leu Glu Thr MET Pro Asp Leu Arg Thr Leu Ser Thr Leu His Thr Glu
AAA CTG GTA GTT TTC CGC ACC GAG CAC AAG GAG CTG CTG CGC CAG CAG ATG TGG
Lys Leu Val Val Phe Arg Thr Glu His Lys Glu Leu Leu Arg Gln Gln MET Trp
594
2162
TCC ATG GAG GAC GCC AAC AAC AGC GAT GCC CAG CAG AAC AAG TCG CCC TCG CCC
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Ser Trp Ala Asp Ala MET Asp Val Glu Ala Ala Lys Ser Pro Leu Gly Ser Val
TCG AGC AGT GAG TCC GCC GAC CTG GAC TAC GCC AGT CCG AGC AGT TCG CAG CCA
Ser Ser Thr Glu Ser Ala Asp Leu Asp Tyr Gly Ser Pro Ser Ser Ser Gln Pro
648
CAG GGC GTG TCT CTG CCC TCG CCG CCT CAG CAA CAG GCC TCG GCT CTG GCC AGC
Gln Gly Val Ser Leu Pro Ser Pro Pro Gln Gln Gln Pro Ser Ala Leu Ala Ser
TCG GCT CCT CTG CTG GCG GCC ACC CTC TCC GGA GGA TGT GCC CTG CGC AAC CGG
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2432
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TCG CAC GCA CAT CTC ACC CAG AAC GGC CTG ACA ATC ACG CCG ATT GTG CGA CAC
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CGC AAC TTG AAT GGG GGA CAC GCG ATG TGC CAG CAA CAG CAG CAG CAC CCA CAA
Arg Asn Leu Asn Gly Gly His Ala MET Cys Gln Gln Gln Gln Gln His Pro Gln
756
G (Dw4925)
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Leu His His His Leu Thr Ala Gly Ala Ala Arg Tyr Arg Lys Leu Asp Ser Pro
Arg
2702
ACG GAT TCG GGC ATT GAG TCG GGC AAC GAG AAG AAC GAG TGC AAG GCG GTG AGT
Thr Asp Ser Gly Ile Glu Ser Gly Asn Glu Lys Asn Glu Cys Lys Ala Val Ser
TCG GGG GGA AGT TCC TCG TGC TCC AGT CCG CGT TCC AGT GTG GAT GAC GCG CTG
Ser Gly Gly Ser Ser Ser Cys Ser Ser Pro Arg Ser Ser Val Asp Asp Ala Leu
810
GAC TGC AGC GAT GCC GCC GCC AAT CAC AAT CAG GTG GTG CAG CAT CCG CAG CTG
Asp Cys Ser Asp Ala Ala Ala Asn His Asn Gln Val Val Gln His Pro Gln Leu
AGT GTG GTG TCC GTG TCA CCA GTT CCC TCG CCC CAG GCC TCC ACC AGC AGC CAT
Ser Val Val Ser Val Ser Pro Val Arg Ser Pro Gln Pro Ser Thr Ser Ser His
CTG AAG CGA CAG ATT GTG GAG GAT ATG CCC GTG CTG AAG CGC GTG CTG CAG GCT
Leu Lys Arg Gln Ile Val Glu Asp MET Pro Val Leu Lys Arg Val Leu Gln Ala
864
2972
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Pro Pro Leu Tyr Asp Thr Asn Ser Leu MET Asp Glu Ala Tyr Lys Pro His Lys
AAA TTC CGG GCC CTG CGG CAT CGC GAG TTC GAG ACC GCC GAG GCG GAT GCC AGC
Lys Phe Arg Ala Leu Arg His Arg Glu Phe Glu Thr Ala Glu Ala Asp Ala Ser
AGT TCC ACT TCC GGC TCG AAC AGC CTG AGT GCC GGC AGT CCG CGG CAG AGC CCA
Ser Ser Thr Ser Gly Ser Asn Ser Leu Ser Ala Gly Ser Pro Arg Gln Ser Pro
918
GTC CCG AAC AGT GTG GCC ACG CCC CCG CCA TCG GCG GCC AGC GCC GCC GCA GGT
Val Pro Asn Ser Val Ala Thr Pro Pro Pro Ser Ala Ala Ser Ala Ala Ala Gly
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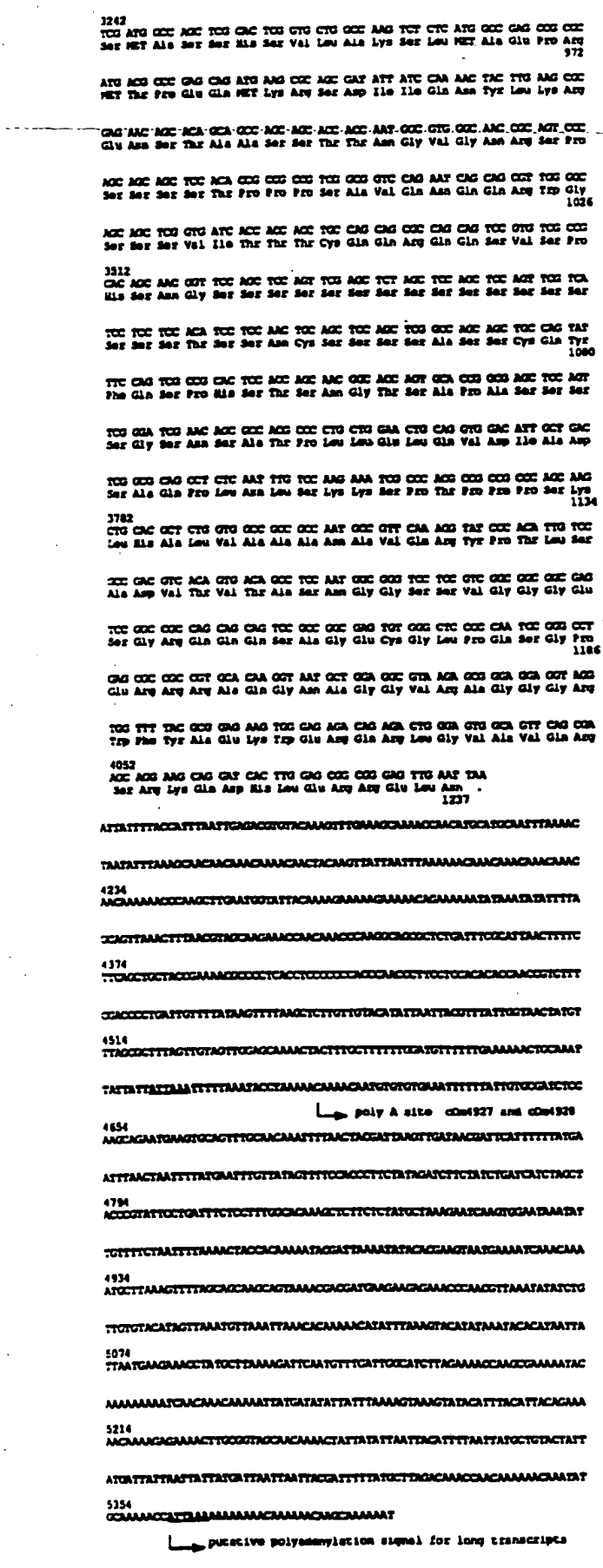
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putative polyadenylation signal for long transcripts

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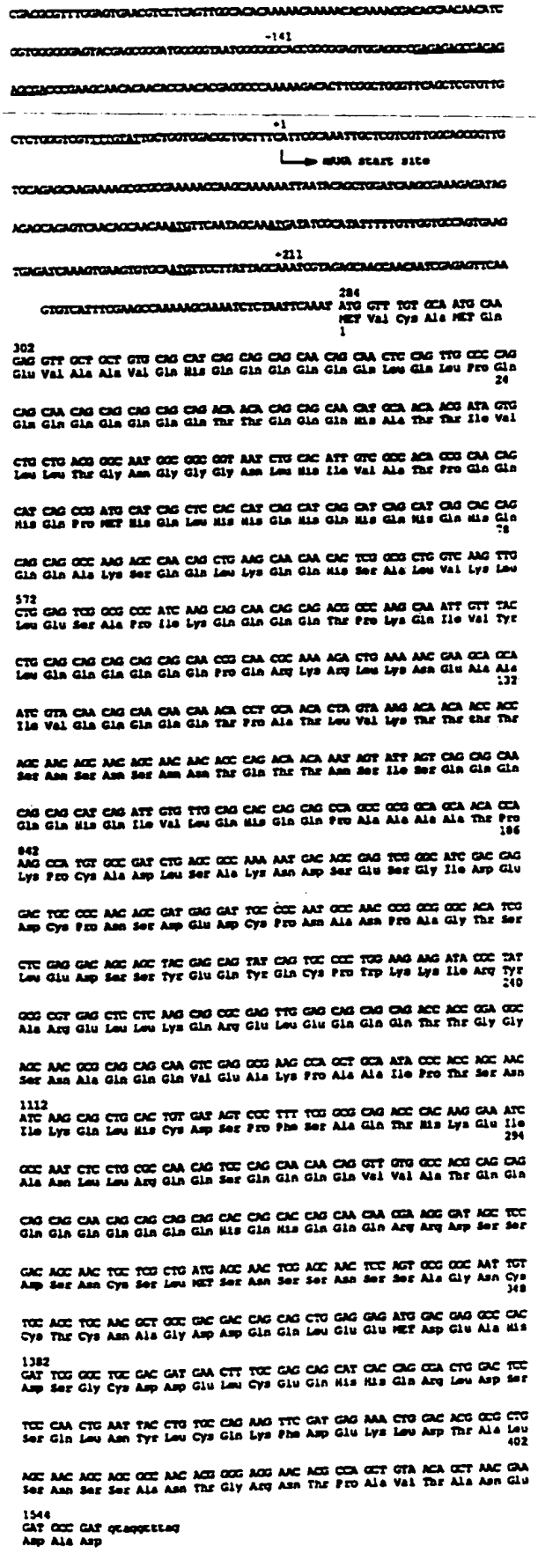
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+1
mRNA start site
-211

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                                                  mRNA start site

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380
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MET Leu MET Ser Ala Asp Ser Ser Asp Ser Ala Lys Thr Ser Val Ile Cys Ser
1

ACG GTG AGT GCC AGC ATG CTA GCA CCA CCA GCT CCA GAA CAG CCC AGC ACC ACA
Thr Val Ser Ala Ser MET Leu Ala Pro Pro Ala Pro Glu Gln Pro Ser Thr Thr

GCA CCA CCC ATT TTG GGG GTA ACA GGT CGA TCT CAC CTG GAA AAT GCC CTG AAA
Ala Pro Pro Ile Leu Gly Val Thr Gly Arg Ser His Leu Glu Asn Ala Leu Lys
                                                                    54
542
CTA CCG CCA AAC ACA AGT GTT TCG GCT TAC TAC CAG CAC AAC AGC AAG CTG GGC
Leu Pro Pro Asn Thr Ser Val Ser Ala Tyr Tyr Gln His Asn Ser Lys Leu Gly

ATG GGC CAG AAT TAC AAT CCG GAA TTC AGG AGC CTG GTA GCA CCT GTC ACA GAT
MET Gly Gln Asn Tyr Asn Pro Glu Phe Arg Ser Leu Val Ala Pro Val Thr Asp

CTG GAT ACT GTG CCA CCC ACA GGT GTG ACC ATG GCG AGT TCT TCG AAT TCT CCC
Leu Asp Thr Val Pro Pro Thr Gly Val Thr MET Ala Ser Ser Ser Asn Ser Pro
                                                                    108

AAC TCC TCC GTC AAG CTG CCC CAC AGC GGC GTG ATC TTT GTC AGC AAA TCG AGT
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GCC GTC AGC ACC ACC GAT GGT CCC ACT GCA GTG TTG CAA CAG CAG CAG CCG CAG
Ala Val Ser Thr Thr Asp Gly Pro Thr Ala Val Leu Gln Gln Gln Gln Pro Gln

812
CAG CAA ATG CCC CAG CAC TTC GAG TCC CTG CCC CAC CAC CAC CCC CAG CAG GAA
Gln Gln MET Pro Gln His Phe Glu Ser Leu Pro His His His Pro Gln Gln Glu
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812
CAG CAA ATG CCC CAG CAC TTC GAG TCC CTG CCC CAC CAC CAC CCC CAG CAG GAA
Gln Gln MET Pro Gln His Phe Glu Ser Leu Pro His His His Pro Gln Gln Glu
162

CAC CAG CCA CAG CAG CAG CAG CAA CAA CAT CAC CTT CAG CAC CAC CCA CAT CCA
His Gln Pro Gln Gln Gln Gln Gln Gln His His Leu Gln His His Pro His Pro

CAT GTG ATG TAT CCG CAC GGA TAT CAG CAG GCC AAT CTG CAC CAC TCG GGT GGT
His Val MET Tyr Pro His Gly Tyr Gln Gln Ala Asn Leu His His Ser Gly Gly

ATT GCT GTG GTT CCG GCG GAT TCG CGT CCC CAG ACT CCC GAG TAC ATC AAG TCC
Ile Ala Val Val Pro Ala Asp Ser Arg Pro Gln Thr Pro Glu Tyr Ile Lys Ser
216

TAC CCA GTT ATG GAT ACA ACT GTG GCT AGT TCG GTA AAG GGG GAA CCA GAA CTC
Tyr Pro Val MET Asp Thr Thr Val Ala Ser Ser Val Lys Gly Glu Pro Glu Leu

GTGAGTTGTG..intron 1..TTCTTTGCAG

1082
AAC ATA GAA TTC GAT GGC ACC ACA GTG CTG TGC CGC GTT TGC GGG GAT AAG GCC
Asn Ile Glu Phe Asp Gly Thr Thr Val Leu Cys Arg Val Cys Gly Asp Lys Ala

GTAAGTTCGT..intron 2..ATCGTTTCAG

TCC GGT TTC CAT TAC GGC GTG CAT TCC TGC GAG GGT TGC AAG GGA TTC TTC CGC
Ser Gly Phe His Tyr Gly Val His Ser Cys Glu Gly Cys Lys Gly Phe Phe Arg
270

CGC TCC ATC CAG CAA AAG ATC CAG TAT CGC CCG TGC ACC AAG AAT CAG CAG TGC
Arg Ser Ile Gln Gln Lys Ile Gln Tyr Arg Pro Cys Thr Lys Asn Gln Gln Cys

AGC ATT CTG CGC ATC AAT CGC AAT CGT TGT CAA TAT TGC CGC CTG AAA AAG TGC
Ser Ile Leu Arg Ile Asn Arg Asn Arg Cys Gln Tyr Cys Arg Leu Lys Lys Cys

GTGAGTACCT..intron 3..CCAATTGCAG

ATT GCC GTG GGC ATG AGT CGC GAT GCT GTG CGT TTT GGA CGC GTG CCG AAG CGC
Ile Ala Val Gly MET Ser Arg Asp Ala Val Arg Phe Gly Arg Val Pro Lys Arg
324

1352
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Glu Lys Ala Arg Ile Leu Ala Ala MET Gln Gln Ser Thr Gln Asn Arg Gly Gln

CAG CGA GCC CTC GCC ACC GAG CTG GAT GAC CAG CCA CGC CTC CTC GCC GCC GTG
Gln Arg Ala Leu Ala Thr Glu Leu Asp Asp Gln Pro Arg Leu Leu Ala Ala Val

CTG CGC GCC CAC CTC GAG ACC TGT GAG TTC ACC AAG GAG AAG GTC TCG GCG ATG
Leu Arg Ala His Leu Glu Thr Cys Glu Phe Thr Lys Glu Lys Val Ser Ala MET
378

GTAAGTCTCA..intron 4..ATTTCTTCAG

CGG CAG CGG GCG CGG GAT TGC CCC TCC TAC TCC ATG CCC ACA CTT CTG GCC TGT
Arg Gln Arg Ala Arg Asp Cys Pro Ser Tyr Ser MET Pro Thr Leu Leu Ala Cys

CCG CTG AAC CCC GCC CCT GAA CTG CAA TCG GAG CAG GAG TTC TCG CAG CGT TTC
Pro Leu Asn Pro Ala Pro Glu Leu Gln Ser Glu Gln Glu Phe Ser Gln Arg Phe

1622
GCC CAC GTA ATT CGC GGC GTG ATC GAC TTT GCC GGC ATG ATT CGC GGC TTC CAG
Ala His Val Ile Arg Gly Val Ile Asp Phe Ala Gly MET Ile Pro Gly Phe Gln
432

CTG CTC ACC CAG GAC GAT AAG TTC ACG CTC CTG AAG GCG GGA CTC TTC GAC GCC
Leu Leu Thr Gln Asp Asp Lys Phe Thr Leu Leu Lys Ala Gly Leu Phe Asp Ala

CTG TTT GTG CGC CTG ATC TGC ATG TTT GAC TCG TCG ATA AAC TCA ATC ATC TGT
Leu Phe Val Arg Leu Ile Cys MET Phe Asp Ser Ser Ile Asn Ser Ile Ile Cys

CTA AAT GGC CAG GTG ATG CGA CGG GAT GCG ATC CAG AAC GGA GCC AAT GCC CGC
Leu Asn Gly Gln Val MET Arg Arg Asp Ala Ile Gln Asn Gly Ala Asn Ala Arg
486

TTC CTG GTG GAC TCC ACC TTC AAT TTC GCG GAG CGC ATG AAC TCG ATG AAC CTG
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CCC GGT TTG CGC AAC CTG GAG CTG ATC GAG AAG ATG TAC TCG CGA CTC AAG GGC
Pro Gly Leu Arg Asn Leu Glu Leu Ile Glu Lys MET Tyr Ser Arg Leu Lys Gly
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Lys Leu Leu Glu Thr MET Pro Asp Leu Arg Thr Leu Ser Thr Leu His Thr Glu

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Lys Leu Val Val Phe Arg Thr Glu His Lys Glu Leu Leu Arg Gln Gln MET Trp
2162 594
TCC ATG GAG GAC GGC AAC AAC AGC GAT GGC CAG CAG AAC AAG TCG CCC TCG GGC
Ser MET Glu Asp Gly Asn Asn Ser Asp Gly Gln Gln Asn Lys Ser Pro Ser Gly

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Ser Trp Ala Asp Ala MET Asp Val Glu Ala Ala Lys Ser Pro Leu Gly Ser Val

TCG AGC ACT GAG TCC GCC GAC CTG GAC TAC GGC AGT CCG AGC AGT TCG CAG CCA
Ser Ser Thr Glu Ser Ala Asp Leu Asp Tyr Gly Ser Pro Ser Ser Ser Gln Pro
648

CAG GGC GTG TCT CTG CCC TCG CCG CCT CAG CAA CAG CCC TCG GCT CTG GCC AGC
Gln Gly Val Ser Leu Pro Ser Pro Pro Gln Gln Gln Pro Ser Ala Leu Ala Ser

TCG GCT CCT CTG CTG GCG GCC ACC CTC TCC GGA GGA TGT CCC CTG CGC AAC CGG
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2432
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702

2432
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702

TCG CAC GCA CAT CTC ACC CAG AAC GGG CTG ACA ATC ACG CCG ATT GTG CGA CAC
Ser His Ala His Leu Thr Gln Asn Gly Leu Thr Il Thr Pro Ile Val Arg His

GTAGTATCTT..intron 5..TTTCTTACAG

CAG CAG CAG CAA CAA CAG CAG CAG CAG ATC GGA ATA CTC AAT AAT GCG CAT TCC
Gln Gln Gln Gln Gln Gln Gln Gln Gln Ile Gly Ile Leu Asn Asn Ala His Ser

CGC AAC TTG AAT GGG GGA CAC GCG ATG TGC CAG CAA CAG CAG CAG CAC CCA CAA
Arg Asn Leu Asn Gly Gly His Ala MET Cys Gln Gln Gln Gln His Pro Gln
756

G (Dm4925)
CTG CAC CAC CAC TTG ACA GCC GGA GCT GCC CGC TAC AGA AAG CTA GAT TCG CCC
Leu His His His Leu Thr Ala Gly Ala Ala Arg Tyr Arg Lys Leu Asp Ser Pro
Arg
2702
ACG GAT TCG GGC ATT GAG TCG GGC AAC GAG AAG AAC GAG TGC AAG GCG GTG AGT
Thr Asp Ser Gly Ile Glu Ser Gly Asn Glu Lys Asn Glu Cys Lys Ala Val Ser

TCG GGG GGA AGT TCC TCG TGC TCC AGT CCG CGT TCC AGT GTG GAT GAT GCG CTG
Ser Gly Gly Ser Ser Ser Cys Ser Ser Pro Arg Ser Ser Val Asp Asp Ala Leu
810

GAC TGC AGC GAT GCC GCC GCC AAT CAC AAT CAG GTG GTG CAG CAT CCG CAG CTG
Asp Cys Ser Asp Ala Ala Ala Asn His Asn Gln Val Val Gln His Pro Gln Leu

AGT GTG GTG TCC GTG TCA CCA GTT CGC TCG CCC CAG CCC TCC ACC AGC AGC CAT
Ser Val Val Ser Val Ser Pro Val Arg Ser Pro Gln Pro Ser Thr Ser Ser His

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Leu Lys Arg Gln Ile Val Glu Asp MET Pro Val Leu Lys Arg Val Leu Gln Ala
864
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Lys Phe Arg Ala Leu Arg His Arg Glu Phe Glu Thr Ala Glu Ala Asp Ala Ser

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Val Pro Asn Ser Val Ala Thr Pro Pro Pro Ser Ala Ala Ser Ala Ala Ala Gly

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Asn Pro Ala Gln Ser Gln Leu His MET His Leu Thr Arg Ser Ser Pro Lys Ala

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1026

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His Ser Asn Gly Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser

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1080

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TCG GGA TCG AAC AGC GCC ACG CCC CTG CTG GAA CTG CAG GTG GAC ATT GCT GAC
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1134

3782
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1237

4052
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1237

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poly A site cDm4927 and cDm4928

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putative polyadenylation signal for long transcripts

B


-141
+1
mRNA start site
+211
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Glu Val Ala Ala Val Gln His Gln Gln Gln Gln Gln Leu Gln Leu Pro Gln
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His Gln Pro MET His Gln Leu His His Gln His Gln His Gln His Gln His Gln
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Ser Asn Ser Asn Ser Asn Asn Thr Gln Thr Thr Asn Ser Ile Ser Gln Gln Gln

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Gln Gln Gln Gln Gln Gln Gln His Gln His Gln Gln Gln Arg Arg Asp Ser Ser

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                                                                    348
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1382
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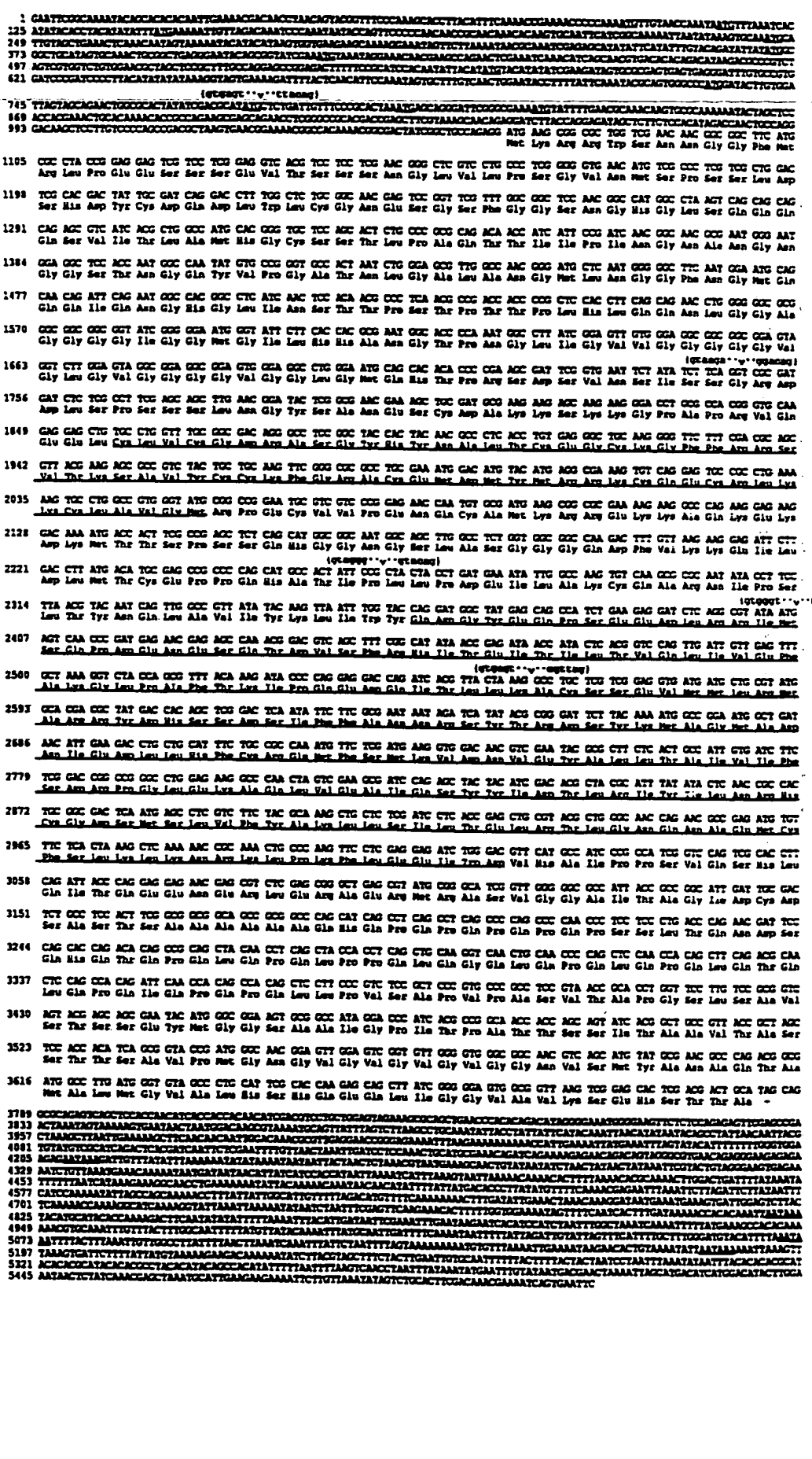
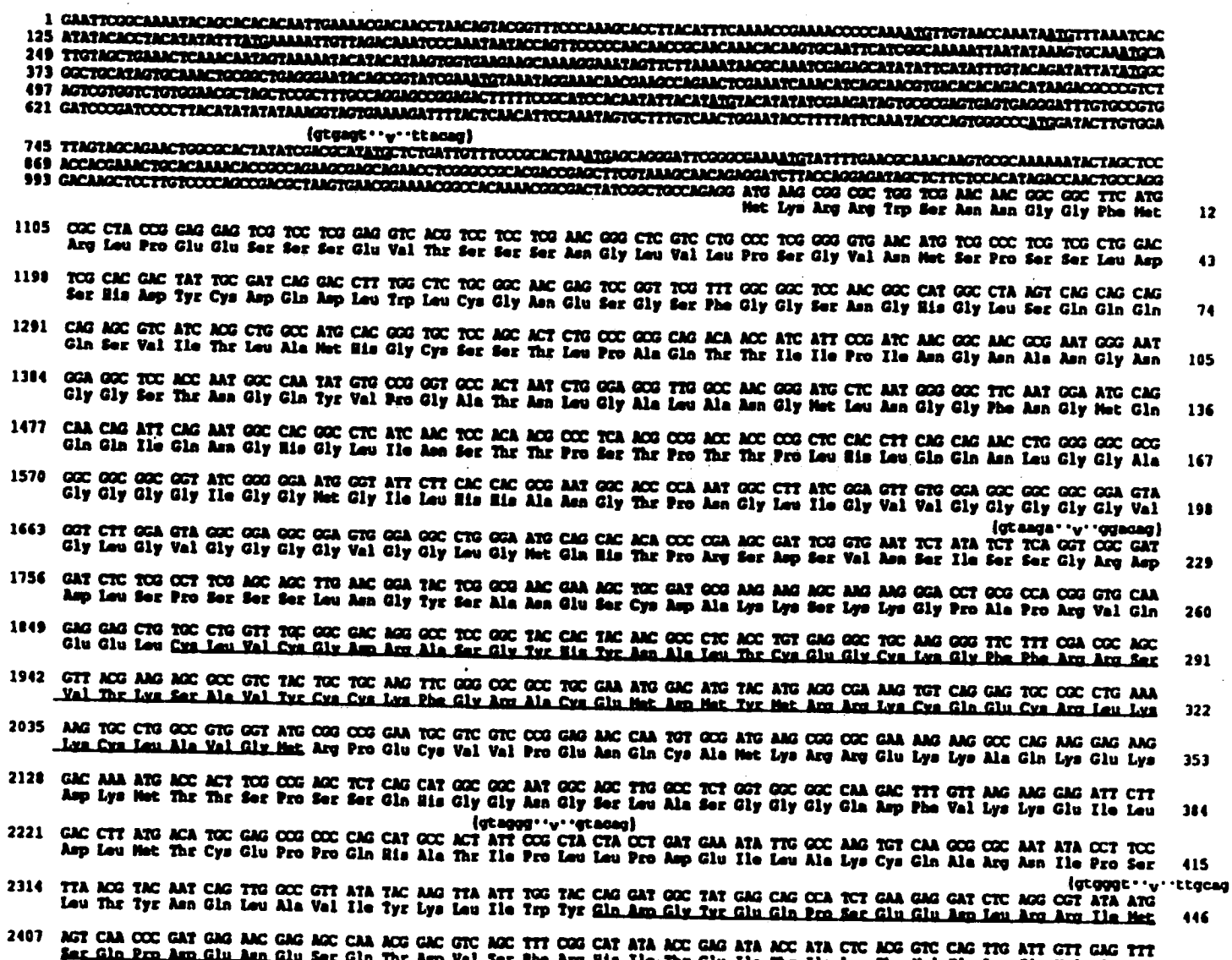
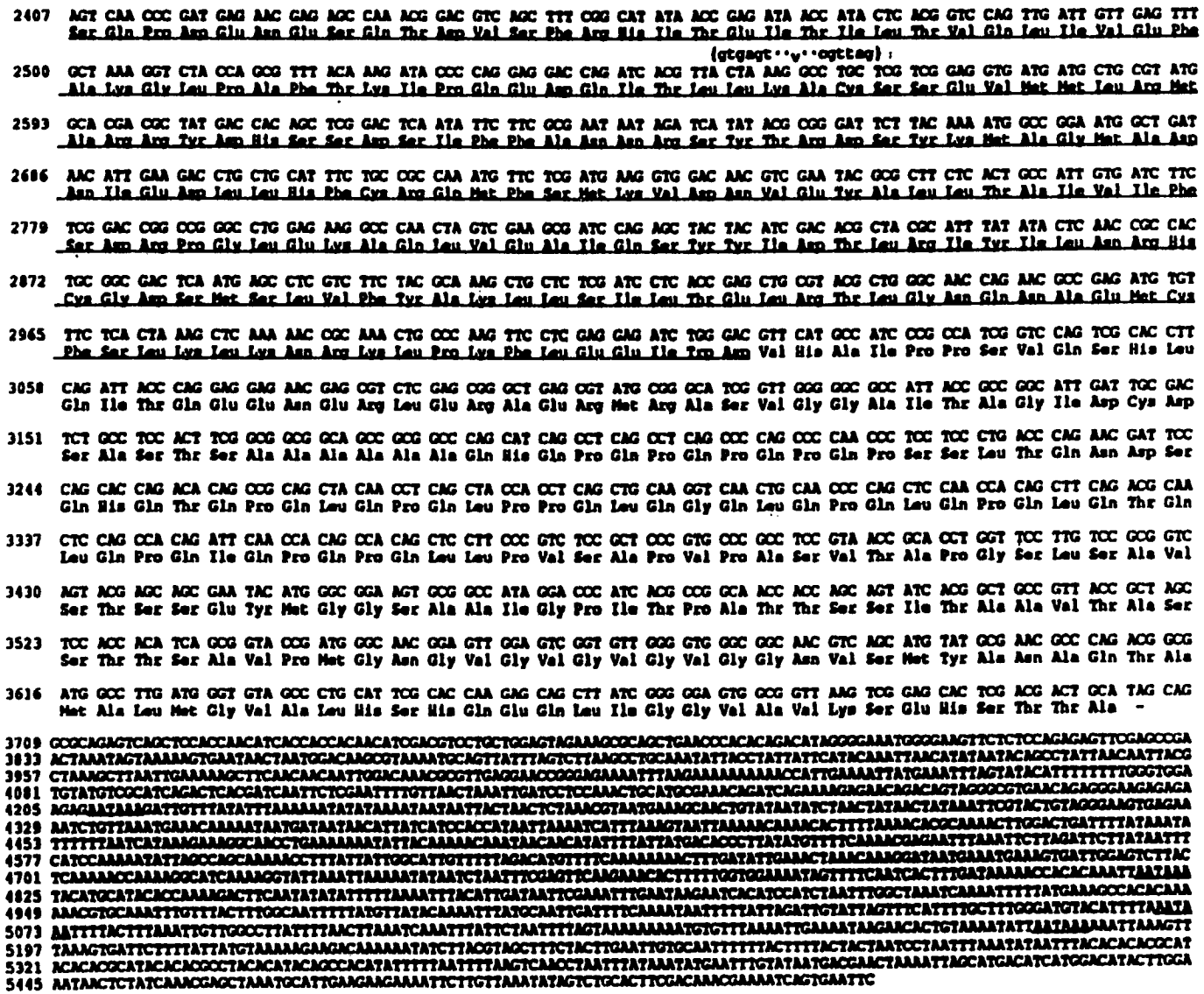
EcR cDNA 3534 bp



EcR cDNA 5534 bp

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Ser Gln Pro Asp Glu Asn Glu Ser Gln Thr Asp Val Ser Phe Arg His Ile Thr Glu Ile Thr Ile Leu Thr Val Glu Leu Ile Val Glu Phe 477
(gtgagt··y··ogttag);
2500 GCT AAA GGT CTA CCA GCG TTT ACA AAG ATA CCC CAG GAG GAC CAG ATC ACG TTA CTA AAG GCC TGC TCG TCG GAG GTG ATG ATG CTG CGT ATG
Ala Lys Gly Leu Pro Ala Phe Thr Lys Ile Pro Gln Glu Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu Arg Met 508
2593 GCA CGA CGC TAT GAC CAC AGC TCG GAC TCA ATA TTC TTC GCG AAT AAT AGA TCA TAT ACG CGG GAT TCT TAC AAA ATG GCC GGA ATG GCT GAT
Ala Arg Arg Tyr Asp His Ser Ser Asp Ser Ile Phe Phe Ala Asn Asn Arg Ser Tyr Thr Arg Asp Ser Tyr Lys Met Ala Gly Met Ala Asp 539
2686 AAC ATT GAA GAC CTG CTG CAT TTC TGC CGC CAA ATG TTC TCG ATG AAG GTG GAC AAC GTC GAA TAC GCG CTT CTC ACT GCC ATT GTG ATC TTC
Asn Ile Glu Asp Leu Leu His Phe Cys Arg Gln Met Phe Ser Met Lys Val Asp Asn Val Glu Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe 570
2779 TCG GAC CGG CCG GGC CTG GAG AAG GCC CAA CTA GTC GAA GCG ATC CAG AGC TAC TAC ATC GAC ACG CTA CGC ATT TAT ATA CTC AAC CGC CAC
Ser Asp Arg Pro Gly Leu Glu Lys Ala Gln Leu Val Glu Ala Ile Gln Ser Tyr Tyr Ile Asp Thr Leu Arg Ile Tyr Ile Leu Asn Arg His 601
2872 TGC GGC GAC TCA ATG AGC CTC GTC TTC TAC GCA AAG CTG CTC TCG ATC CTC ACC GAG CTG CGT ACG CTG GGC AAC CAG AAC GCC GAG ATG TGT
Cys Gly Asp Ser Met Ser Leu Val Phe Tyr Ala Lys Leu Leu Ser Ile Leu Thr Glu Leu Arg Thr Leu Gly Asn Gln Asn Ala Glu Met Cys 632
2965 TTC TCA CTA AAG CTC AAA AAC CGC AAA CTG CCC AAG TTC CTC GAG GAG ATC TGG GAC GTT CAT GCC ATC CGG CCA TCG GTC CAG TCG CAC CTT
Phe Ser Leu Lys Leu Lys Asn Arg Lys Leu Pro Lys Phe Leu Glu Glu Ile Trp Asp Val His Ala Ile Pro Pro Ser Val Gln Ser His Leu 663
3058 CAG ATT ACC CAG GAG GAG AAC GAG CGT CTC GAG CGG GCT GAG CGT ATG CGG GCA TCG GTT GGG GGC GCC ATT ACC GCC GGC ATT GAT TGC GAC
Gln Ile Thr Gln Glu Glu Asn Glu Arg Leu Glu Arg Ala Glu Arg Met Arg Ala Ser Val Gly Gly Ala Ile Thr Ala Gly Ile Asp Cys Asp 694
3151 TCT GCC TCC ACT TCG GCG GCG GCA GCC GCG GCC CAG CAT CAG CCT CAG CCT CAG CCC CAG CCC CAA CCC TCC TCC CTG ACC CAG AAC GAT TCC
Ser Ala Ser Thr Ser Ala Ala Ala Ala Ala Ala Gln His Gln Pro Gln Pro Gln Pro Gln Pro Gln Pro Ser Ser Leu Thr Gln Asn Asp Ser 725
3244 CAG CAC CAG ACA CAG CCG CAG CTA CAA CCT CAG CTA CCA CCT CAG CTG CAA GGT CAA CTG CAA CCC CAG CTC CAA CCA CAG CTT CAG ACG CAA
Gln His Gln Thr Gln Pro Gln Leu Gln Pro Gln Leu Pro Pro Gln Leu Gln Gly Gln Leu Gln Pro Gln Leu Gln Pro Gln Leu Gln Thr Gln 756
3337 CTC CAG CCA CAG ATT CAA CCA CAG CCA CAG CTC CTT CCC GTC TCC GCT CCC GTG CCC GCC TCC GTA ACC GCA CCT GGT TCC TTG TCC GCG GTC
Leu Gln Pro Gln Ile Gln Pro Gln Pro Gln Leu Leu Pro Val Ser Ala Pro Val Pro Ala Ser Val Thr Ala Pro Gly Ser Leu Ser Ala Val 787
3430 AGT ACG AGC AGC GAA TAC ATG GGC GGA AGT GCG GCC ATA GGA CCC ATC ACG CCG GCA ACC ACC AGC AGT ATC ACG GCT GCC GTT ACC GCT AGC
Ser Thr Ser Ser Glu Tyr Met Gly Gly Ser Ala Ala Ile Gly Pro Ile Thr Pro Ala Thr Thr Ser Ser Ile Thr Ala Ala Val Thr Ala Ser 818
3523 TCC ACC ACA TCA GCG GTA CCG ATG GGC AAC GGA GTT GGA GTC GGT GTT GGG GTG GGC GGC AAC GTC AGC ATG TAT GCG AAC GCC CAG ACG GCG
Ser Thr Thr Ser Ala Val Pro Met Gly Asn Gly Val Gly Val Gly Val Gly Val Gly Gly Asn Val Ser Met Tyr Ala Asn Ala Gln Thr Ala 849
3616 ATG GCC TTG ATG GGT GTA GCC CTG CAT TCG CAC CAA GAG CAG CTT ATC GGG GGA GTG GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG CAG
Met Ala Leu Met Gly Val Ala Leu His Ser His Gln Glu Gln Leu Ile Gly Gly Val Ala Val Lys Ser Glu His Ser Thr Thr Ala - 878
3709 GCGCAGAGTCAGCTCCACCAACATCACCACCACAACATGGACGTCCTGCTGGAGTAGAAAGCGCAGCTGAACCCACACAGACATAGGGGAAATGGGGAAGTTCTCTCCAGAGAGTTCGAGCCGA
3833 ACTAAATAGTAAAAAGTGAATAACTAATGGACAAGCGTAAAATGCAGTTATTTAGTCTTAAGCCTGCAAATATTACCTATTATTCATACAAATTAACATATAATACAGCCTATTAACAATTACG
3957 CTAAAGCTTAATTGAAAAAGCTTCAACAACAATTGGACAAACGCGTTGAGGAACCGGGAGAAAATTTAAGAAAAAAAAAAACCATTGAAAATTATGAAATTTAGTATACATTTTTTTTGGGTGGA
4081 TGTATGTCGCATCAGACTCACGATCAATTCTCGAATTTTGTTAACTAAATTGATCCTCCAAACTGCATGCGAAACAGATCAGAAAAGAGAACAGACAGTAGGGCGTGAACAGAGGAAGAGAGA
4205 AGAGAATAAAGATTGTTTATATTTAAAAAATATATAAAATAATAATTACTAACTCTAAACGTAATGAAACAACTGTATAATATCTAACTATAACTATAAATTCGTACTGTAGGGAAGTGAGAA
4329 AATCTGTTAAATGAAACAAAAATAATGATAATAACATTATCATCCACCATAATTAAAATCATTTAAAGTAATTAAAAACAAAACACTTTTAAAACACGCAAAACTTGGACTGATTTTATAAATA
4453 TTTTTTAATCATAAAGAAAGCCAACCTGAAAAAAATATTACAAAAACAAATAACAACATATTTTATTATGACACCCTTATATGTTTTCAAAACGAGAATTTAAATTCTTAGATTCTTATAATTT
4577 CATCCAAAAATATTAGCCAGCAAAAACCTTTATTATTGGCATTGTTTTAGACATGTTTTCAAAAAAAACTTTGATATTGAAACTAAACAAAGGATAATGAAATGAAAGTGATTGGAGTCTTAC
4701 TCAAAAACCAAAAGGCATCAAAAGGTATTAAATTAAAAATATAATCTAATTTGGAGTTCAAGAAACACTTTTTGGTGGAAAATAGTTTTCAATCACTTGATAAAAACCACACAAATTAATAAA
4825 TACATGCATACACCAAAAGACTTCAATATATATTTTTAAAATTTACATTGATAATTCGAAATTTGAATAAGAATCACATCCATCTAATTTGGCTAAATCAAAATTTTTATGAAAGCCACACAAA
4949 AAACGTGCAAATTTGTTTACTTTGGCAATTTTATGTTATACAAAATTTATGCAATGATTTCAAAATAATTTTATTAGATTGTATTAGTTCATTTGCTTTGGGATGTACATTTTAAATA
5073 AATTTTACTTTAAATTGTTGGCCTTATTTTAACTTAAATCAAATTTATTCTAATTTTAGTAAAAAAAAATGTGTTTAAAATTGAAAATAAGAACACTGTAAAATATTAATAAAAATTAAAGTT
5197 TAAAGTGATTCTTTTATTATGTAAAAAGAAGACAAAAAATATCTTACGTAGCTTTCTACTTGAATTGTGCAATTTTTTACTTTTACTACTAATCCTAATTTAAATATAATTTACACACACGCAT
5321 ACACACGCATACACACGCCTACACATACAGCCACATATTTTTAATTTTAAGTCAACCTAATTTATAAATATGAATTTGTATAATGACGAACTAAAATTAGCATGACATCATGGACATACTTGGA
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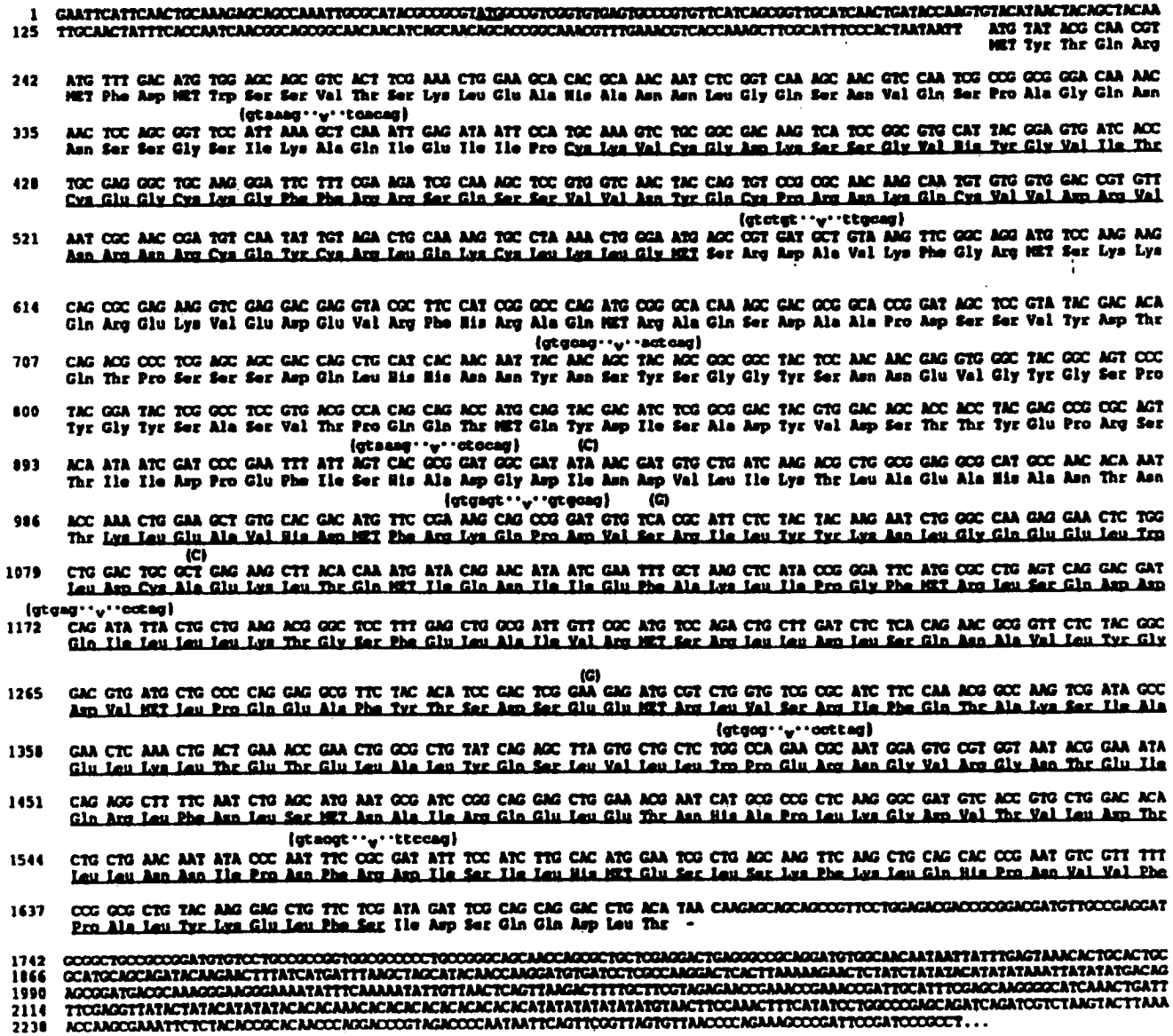
cDNA DHR3-9 4.2 kb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125 TTGCAACTATTTCACCAATCAACCGCCAGCCGCAACAACATCAGCAACAGCACCGGCAAACGTTTGAAACGTCACCAAAGCTTCGCATTTCCCACTAATAATT ATG TAT ACG CAA CGT
MET Tyr Thr Gln Arg 5
242 ATG TTT GAC ATG TGG AGC AGC GTC ACT TCG AAA CTG GAA GCA CAC GCA AAC AAT CTC GGT CAA AGC AAC GTC CAA TCG CCG GCG GGA CAA AAC
MET Phe Asp MET Trp Ser Ser Val Thr Ser Lys Leu Glu Ala His Ala Asn Asn Leu Gly Gln Ser Asn Val Gln Ser Pro Ala Gly Gln Asn 36
(gtaaag··y··tcacag)
335 AAC TCC AGC GGT TCC ATT AAA GCT CAA ATT GAG ATA ATT CCA TGC AAA GTC TGC GGC GAC AAG TCA TCC GGC GTG CAT TAC GGA GTG ATC ACC
Asn Ser Ser Gly Ser Ile Lys Ala Gln Ile Glu Ile Ile Pro Cys Lys Val Cys Gly Asp Lys Ser Ser Gly Val His Tyr Gly Val Ile Thr 67
428 TGC GAG GGC TGC AAG GGA TTC TTT CGA AGA TCG CAA AGC TCC GTG GTC AAC TAC CAG TGT CCG CGC AAC AAG CAA TGT GTG GTG GAC CGT GTT
Cys Glu Gly Cys Lys Gly Phe Phe Arg Arg Ser Gln Ser Ser Val Val Asn Tyr Gln Cys Pro Arg Asn Lys Gln Cys Val Val Asp Arg Val 98
(gtctgt··y··ttgcag)
521 AAT CGC AAC CGA TGT CAA TAT TGT AGA CTG CAA AAG TGC CTA AAA CTG GGA ATG AGC CGT GAT GCT GTA AAG TTC GGC AGG ATG TCC AAG AAG
Asn Arg Asn Arg Cys Gln Tyr Cys Arg Leu Gln Lys Cys Leu Lys Leu Gly MET Ser Arg Asp Ala Val Lys Phe Gly Arg MET Ser Lys Lys 129
614 CAG CGC GAG AAG GTC GAG GAC GAG GTA CGC TTC CAT CGG GCC CAG ATG CGG GCA CAA AGC GAC GCG GCA CCG GAT AGC TCC GTA TAC GAC ACA
Gln Arg Glu Lys Val Glu Asp Glu Val Arg Phe His Arg Ala Gln MET Arg Ala Gln Ser Asp Ala Ala Pro Asp Ser Ser Val Tyr Asp Thr 160
(gtgcag··y··actcag)
707 CAG ACG CCC TCG AGC AGC GAC CAG CTG CAT CAC AAC AAT TAC AAC AGC TAC AGC GGC GGC TAC TCC AAC AAC GAG GTG GGC TAC GGC AGT CCC
Gln Thr Pro Ser Ser Ser Asp Gln Leu His His Asn Asn Tyr Asn Ser Tyr Ser Gly Gly Tyr Ser Asn Asn Glu Val Gly Tyr Gly Ser Pro 191
800 TAC GGA TAC TCG GCC TCC GTG ACG CCA CAG CAG ACC ATG CAG TAC GAC ATC TCG GCG GAC TAC GTG GAC AGC ACC ACC TAC GAG CCG CGC AGT
Tyr Gly Tyr Ser Ala Ser Val Thr Pro Gln Gln Thr MET Gln Tyr Asp Ile Ser Ala Asp Tyr Val Asp Ser Thr Thr Tyr Glu Pro Arg Ser 222
(gtaaag··y··ctccag) (C)
893 ACA ATA ATC GAT CCC GAA TTT ATT AGT CAC GCG GAT GGC GAT ATA AAC GAT GTG CTG ATC AAG ACG CTG GCG GAG GCG CAT GCC AAC ACA AAT
Thr Ile Ile Asp Pro Glu Phe Ile Ser His Ala Asp Gly Asp Ile Asn Asp Val Leu Ile Lys Thr Leu Ala Glu Ala His Ala Asn Thr Asn 253
(gtgagt··y··gtgcag) (G)
986 ACC AAA CTG GAA GCT GTG CAC GAC ATG TTC CGA AAG CAG CCG GAT GTG TCA CGC ATT CTC TAC TAC AAG AAT CTG GGC CAA GAG GAA CTC TGG
Thr Lys Leu Glu Ala Val His Asp MET Phe Arg Lys Gln Pro Asp Val Ser Arg Ile Leu Tyr Tyr Lys Asn Leu Gly Gln Glu Glu Leu Trp 284
(C)
1079 CTG GAC TGC GCT GAG AAG CTT ACA CAA ATG ATA CAG AAC ATA ATC GAA TTT GCT AAG CTC ATA CCG GGA TTC ATG CGC CTG AGT CAG GAC GAT
Leu Asp Cys Ala Glu Lys Leu Thr Gln MET Ile Gln Asn Ile Ile Glu Phe Ala Lys Leu Ile Pro Gly Phe MET Arg Leu Ser Gln Asp Asp 315
(gtgag··y··cctag)
1172 CAG ATA TTA CTG CTG AAG ACG GGC TCC TTT GAG CTG GCG ATT GTT CGC ATG TCC AGA CTG CTT GAT CTC TCA CAG AAC GCG GTT CTC TAC GGC
Gln Ile Leu Leu Leu Lys Thr Gly Ser Phe Glu Leu Ala Ile Val Arg MET Ser Arg Leu Leu Asp Leu Ser Gln Asn Ala Val Leu Tyr Gly 346
(G)
1265 GAC GTG ATG CTG CCC CAG GAG GCG TTC TAC ACA TCC GAC TCG GAA GAG ATG CGT CTG GTG TCG CGC ATC TTC CAA ACG GCC AAG TCG ATA GCC
Asp Val MET Leu Pro Gln Glu Ala Phe Tyr Thr Ser Asp Ser Glu Glu MET Arg Leu Val Ser Arg Ile Phe Gln Thr Ala Lys Ser Ile Ala 377
(gtgcg··y··ccttag)
1358 GAA CTC AAA CTG ACT GAA ACC GAA CTG GCG CTG TAT CAG AGC TTA GTG CTG CTC TGG CCA GAA CGC AAT GGA GTG CGT GGT AAT ACG GAA ATA
Glu Leu Lys Leu Thr Glu Thr Glu Leu Ala Leu Tyr Gln Ser Leu Val Leu Leu Trp Pro Glu Arg Asn Gly Val Arg Gly Asn Thr Glu Ile 408
1451 CAG AGG CTT TTC AAT CTG AGC ATG AAT GCG ATC CGG CAG GAG CTG GAA ACG AAT CAT GCG CCG CTC AAG GGC GAT GTC ACC GTG CTG GAC ACA
Gln Arg Leu Phe Asn Leu Ser MET Asn Ala Ile Arg Gln Glu Leu Glu Thr Asn His Ala Pro Leu Lys Gly Asp Val Thr Val Leu Asp Thr 439
(gtaagt··y··ttccag)
1544 CTG CTG AAC AAT ATA CCC AAT TTC CGC GAT ATT TCC ATC TTG CAC ATG GAA TCG CTG AGC AAG TTC AAG CTG CAG CAC CCG AAT GTC GTT TTT
Leu Leu Asn Asn Ile Pro Asn Phe Arg Asp Ile Ser Ile Leu His MET Glu Ser Leu Ser Lys Phe Lys Leu Gln His Pro Asn Val Val Phe 470
1637 CCG GCG CTG TAC AAG GAG CTG TTC TCG ATA GAT TCG CAG CAG GAC CTG ACA TAA CAAGAGCAGCAGCCGTTCCTGGAGACGACCCCCGGACGATGTTCCCGAGGAT
Pro Ala Leu Tyr Lys Glu Leu Phe Ser Ile Asp Ser Gln Gln Asp Leu Thr - 487
1742 GCCGCTGCCCCCCGGATGTGTCCTGCCCCCGGTGGCCCCCCCTGCCCGGCAGCAACCAGCGCTGCTGGAGGACTGAGGGCCGCAGGATGTGGCAACAATAATTATTTGAGTAAACACTGCACTGC
1866 GCATGCAGCAGATACAAGAACTTTATCATGATTTAAGCTAGCATACAACCAAGGATGTGATCCTCGCCAAGGACTCACTTAAAAAGAACTCTATCTATATACATATATAAATTATATATGACAG
1990 AGCGGATGACCCAAAGGGAAGGGAAAATATTTCAAAAATATTGTTAACTCAGTTAAGACTTTTGCTTCGTAGAGAACCGAAACCGAAACCGATTCCATTTCGAGCAAGGGGCATCAAACTGATT
2114 TTCGAGGTTATACTATACATATATACACACAAACACACACACACACACACATATATATATATGTAACTTCCAAACTTTCATATCCTGGCCCGAGCAGATCAGATCGTCTAAGTACTTAAA
2238 ACCAAGCGAAATTCTCTACACCGCACAACCCAGGACCCGTAGACCCCAATAATTCAGTTCGGTTAGTGTTAACCCCAGAAAGCCCGATTCCGATCCCGCCT...

A

		●● ●
DHR3	51	CKVCGDKSSGVHYGVITCEGCKGFFRRSQSSVV--NYQCPRNKQCVVDRVNRNRCQYCRLQKCLKLGM
EcR	264	CLVCGDRASGYHYNALTCEGCKGFFRRSVTKSA--VYCCKFGRACEMDMYMRRKCQECRLKKCLAVGM
E75A	245	CRVCGDKASGFHYGVHSCEGCKGFFRRSIQQKI-QYRPCTKNQQCSILRINRNRCQYCRLKKCIAVGM
kni	5	CKVCGEPAAGFHFGAFTCEGCKSFFGRSYNNIS-TISECKNEGKCIIDKKNRTTCKACRLRKCYNVGM
hRARα	58	CFVCQDKSSGYHYGVSACEGCKGFFRRSIQKNM--VYTCHRDKNCIINKVTRNRCQYCRLQKCFEVGM
hTRβ	102	CVVCGDKATGYHYRCITCEGCKGFFRRTIQKNLHPSYSCKYEGKCVIDKVTRNQCQECRFKKCIYVGM
hVDR	24	CGVCGDRATGFHFNAMTCEGCKGFFRRSMKRKA--LFTCPFNGDCRITKDNRRHCQACRLKRCVDIGM
cOUP-TF		CVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRNL--TYTCRANRNCPIDQHHRNQCQYCRLKKCLKVGM
hERR1	175	CLVCGDVASGYHYGVASCEACKAFFKRTIQGSI--EYSCPASNECEITKRRRKACQACRFTKCLRVGM
hERR2	103	CLVCGDIASGYHYGVASCEACKAFFKRTIQGNI--EYSCPATNECEITKRRRKSCQACRFMKCLKVGM
hER	185	CAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHN--DYMCPATNQCTIDKNRRKSCQACRLRKCYEVGM
hGR	421	CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQH--NYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGM
hMR	603	CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGQH--NYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
hPR	567	CLICGDEASGCHYGVLTCGSCKVFFKRAMEGQH--NYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGM

B

E1

DHR3	255	KLEAVHDMFRKQPDVSRILYYKNLGQEKLWLDCAEKLTQMIQNIIEFAKLLPGFMRLSQDDQILLLKTGSFELAIVRMSRL--LDLSQNAVLYGDVMLPQEAFYTS--DSEEMRLVSRIFQTAKSIAEL
EcR	431	QDGYEQPSEEDLRRIMSQPDENESQTDVSFRHITEITILTVQLIVEFAKGLPAFTKIPQEDQITLLKACSSEVMMLRMARR--YDHSSDSIFFANNRSYTRDSYKM-AGMADNIEDLLHFCRQMFSMKV
E75A	380	QRARDCPSYSMPTLLACPLNPAPELQSEQEF--SQRFAHVIRGVIDFAGMIPGFQLLTQDDKFTLLKAGLFDALFVRLICM--FDSSINSIICLN-GQVMRRDAIQ-NGANARFLVDSTFNFAERMNSM
hRARα	170	PALCQLGKYTTNNSSEQRVSLDIDL--WDKF--SELSTKCIIKTVEFAKQLPGFTTLTIADQITLLKAACLDILILRICTR--YTPEQDTMTFSDGLTLNRTQMHN-AGFGPLTDLVFAFANQLLPLEM
hTRβ	238	PKFLPEDIGQAPIVNAPEGG-KVDLEAFSHF--TKIITPAITRVVDFAKKLPMFCELPCEDQIILLKGCCMEIMSLRAAVR--YDPESETLTLNGEMAVIRGQLKN-GGLGVVSDAIFDLGMSLSSFNL
hVDR	198	DSSSFSNLDLSEEDSDDPSVTLELSQLSMLPHLADLVSYSIQKVIGFAKMIPGFRDLTSEDQIVLLKSSAIEVIMLRSNES--FTMDDMSWTCGNQDYKYRVSDVTKAGHSLELIEPLIKFQVGLKKLN
cOUP-TF		GYISLLLRAEPYPTSRYGSQCMQPNNIMGIENICELAARLLFSAVEWARNIPFFPDLQITDQVSLLRLTWSELFVLNAAQCSMPLHVAPLLAAAGLHASPMSADRV-VAFMDH----------IRIFQEN
hERR1	294	LVSHLLV-VEPEKLYAMPDPAGPDGHLPAVATLCDLFDREIVVTISWAKSIPGFSSLSLSDQMSVLQSVWMEVLVLGVAQRSLPLQDE--LAFAEDLVLDEEGARA-AGLGEL----------GAALLQL
hERR2	211	IVSYLLV-AEPDKLYAMPPDDVPEGDIKALTTLCDLADRELVFLISWAKHIPGFSNLTLGDQMSLLQSAWMEILILGIVYRSLPYDDK--LAYAEDYIMDEEHSRL-VGLLEL----------YRAILQL
hER	315	MVSALLD-AEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHP-VKLL-FAPNLLLDRNQGKC-VEGMVE---IFDMLLATSSRFR
hGR	531	TLVSLLEVIEPEVLYAGYDSSVPDSTWRIMTTLNMLGGRQVIAAVKWAKAIPGFRNLHLDDQMTLLQYSWMFLMAFALGWRSYRQSSANLLCFAPDLIINE-QRMT-LPCMYDQCKH-----MLYVSSE
hMR	737	SPVMVLENIEPEIVYAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLEDQITLIQYSWMCLSSFALSWRSYKHTNSQFLYFAPDLVFNE-EKMH-QSAMYELCQG-----MHQISLQ
hPR	686	PLINLLMSIEPDVIYAGHDNTKPDTSSSLLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSGQMLYFAPDLILNE-QRMK-ESSFYSLCLT-----MWQIPQE

E2 E3

DHR3	380	KL--------TETELALYQSLVLLMPE-RNGVRGNTEIQRLFNLSMNAIRQ-------------------ELETNHAPLKGDVTVLDTLLNNIPNFRDISILHMESLSKFKLQHPN----VVFPALYKELFS
EcR	557	----------DNVEYALLTAIV-IFSD-RPGLEKAQLVEAIQSYYIDTLRI---------------YILNR---HCGDSMSLVFYAKLLSILTELRTLGNQNAEMCFSLKLKNRK------LPKFLEEIWD
E75A	503	NL--------TDAEIGLFCAIVLITPD-RPGLRNLELIEKMYSRLKGCLQ----------------YIVAQ-----NRPDQPEFLAKLLETMPDLRTLSTLHTEKL-------------VVFRTEHKELLR
hRAR	292	----------DDAETGLLSAICLICGD-RQDLEQPDRVDMLQEPLLEALKV---------------Y-VRK-----RRPSRPHMFPKMLMKITDLRSISAKGAERVITLKMEIPGSM----PPLIQEMLEN
hTRβ	361	----------DDTEVALLQAVLLMSSD-RPGLACVERIEKYQDSFLLAFEH---------------YINYR------KHHVTHFWPKLLMKVTDLRMIGACHASRFLHMKVECPTELL---PPLFLEVFED
hVDR	325	LH---------EEEHVLLMAICIVSPD-RPGVQDAALIEAIQDRLSNTLQT---------------YIRCRHPPPGSHLLYAKMIQKLA----DLRSLNDDHSKQYRCLSFQ-PEC-SMKLTPLVLEVFGN
cOUP-TF		VEKLKALHVDSAEYSCLKAIVLFTSD-ACGLSDAAHIESLQEKSQCALEE----------------------YVRSQ-YPNQPSRFGKLLLRLPSLRTVSSSVIEQLFFVRLVGKTPIE-TLIRDMLLSGSS
hERR1	410	VRRLQALRLEREEYVLLKALALANSD--------SVHIE-DEPRLWSSCE-KLLHEALLEYE---------AGRAGPGGGAERRRAGRLLLTLPLLRQTAGKVLAHFYGVKLEGKVPMH-KLFLEMLEAMMD
hERR2	328	VRRYKKLKVEKEEFVMLKALALANSD--------SMYIENLEAVQKLQ---DLLHEALQDYE------------LSQRHEEPRRAGKLLLTLPLLRQTAAKAVQHFYSVKLQGKVPMH-KLFLEMLEAKV
hER	437	MMNLQ------GEEFVCLKSIILLNSG--------VYTFLSSTLKSLE----EKDHIHRVLDKITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLY-DLLLEMLDAHRL
hGR	653	LHRLQ---VSYEEYLCMKTLLLLSSVPKDGL------------KSQELFDEIRMTYIKELGK---AIVKREGNSSQNWQRFYQ-LTKLLDSMHEVVENLLN------YCFQTFLD-KTMSIEFPEMLAEIIT
hMR	859	FVRLQ----TFEEYTIMKVLLLLSTIPKDGL------------KSQAAFEEMRTNYIKELRK---MVTKCPNNSGQSWQRFYQ-LTKLLDSMHDLVSDLLE------FCFYTFRESHALKVEFPAMLVEIIS
hPR	808	FVKLQ---VSQEEFLCMKVLLLLNTIPLEGL------------RSQTQFEEMRSSYIRELIK---AIGLRQKGVVSSSQRFYQ-LTKLLDNLHDLVKQ------LHLYCLNTFIQSRALSVEFPEMMSEVIA

C

	D H R 3	E c R	E 7 5 A	k n i	h R A R α	h T R β	h V D R	c O U P	h E R R 1	h E R R 2	h E R	h G R	h M R	h P R
DHR3		19	24		18	18	17	14	13	14	15	13	16	15
EcR	55		23		24	29	25	20	16	15	17	14	14	13
E75A	64	55			23	20	22	16	15	15	13	14	12	15
kni	47	48	48											
hRARα	65	55	62	45		33	22	18	18	18	17	14	15	16
hTRβ	56	59	58	52	62		22	16	18	17	19	14	18	15
hVDR	50	58	55	53	47	52		17	20	19	15	13	15	13
cOUP	62	58	58	50	61	59	50		29	30	24	21	19	20
hERR1	48	53	54	43	53	56	45	51		63	32	25	23	25
hERR2	49	51	54	43	54	57	45	54	91		33	25	21	25
hER	53	52	58	52	59	53	47	55	69	72		27	25	27
hGR	47	48	44	45	45	44	41	48	57	57	58		57	54
hMR	52	50	47	47	48	45	44	52	59	59	58	94		56
hPR	48	47	44	45	44	42	42	45	54	54	56	91	91	

A

DHR3	51	CKVCGDKSSGVHYGVITCEGCKGFFRRSQSSVV--NYQCPRNKQCVVDRVNRNRCQYCRLQKCLKLGM
EcR	264	CLVCGDRASGYHYNALTCEGCKGFFRRSVTKSA--VYCCKFGRACEMDMYMRRKCQECRLKKCLAVGM
E75A	245	CRVCGDKASGFHYGVHSCEGCKGFFRRSIQQKI-QYRPCTKNQQCSILRINRNRCQYCRLKKCIAVGM
kn1	5	CKVCGERAAGFHFGAFTCEGCKSFFGRSYHNIS-TISECKNEGKCIIDKKNRTTCKACRLRKCYNVGM
hRARα	58	CFVCQDKSSGYHYGVSACEGCKGFFRRSIQKNM--VYTCHRDKNCIINKVTRNRCQYCRLQKCFEVGM
hTRβ	102	CVVCGDKATGYHYRCITCEGCKGFFRRTIQKNLHPSYSCKYEGKCVIDKVTRNQCQECRFKKCIYVGM
hVDR	24	CGVCGDRATGFHFNAMTCEGCKGFFRRSMKRKA--LFTCPFNGDCRITKDNRNRCQACRLKRCVDIGM
cOUP-TF		CVVCGDKSSGKHYGQFTCEGCKSFFKRSVRRNL--TYTCRANRNCPIDQHHRNQCQYCRLKKCLAVGM
hERR1	175	CLVCGDVASGYHYGVASCEACKAFFKRTIQGSI--EYSCPASNECEITKRRRKACQACRFTKCLRVGM
hERR2	103	CLVCGDIASGYHYGVASCEACKAFFKRTIQGNI--EYSCPATNECKITKRRRKSCQACRFMKCLKVGM
hER	185	CAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHN--DYMCPATNQCTIDKNRRKSCQACRLRKCYEVGM
hGR	421	CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQH--NYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGM
hMR	603	CLVCGDEASGCHYGVVTCGSCKVFFKRAVEGQH--NYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGM
hPR	567	CLICGDEASGCHYGVLTCGSCKVFFKRAMEGQH--NYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGM

B

E1

DER3	255	KLKAVHDMFTKQPDVSRILYYGNLAQKLWLDCAEKLTQMIQNIIEFAKLLPGFTGLSQDDQILLLKTGSFELAIVRMSRLLDLS
EcR	431	QDGYEQPSEEDLARIMSQPDEMKSQTDVSFRHITELTTILTVQLIVEFAKGLPAFTKIPQEDQITLLKACSSEVMMLRMARRYDES
E75A	380	QRARDCPSYSMPTLLACPLMPAPELQSEQMF--SQRFAEVIRGVIDFAGMIPGFQLLIQDDKFTLLKAGLFDALFVRLICM--FDP"
hRARα	170	PALCQLGKYTTNNSSEQRVSLDIDL--WDKF--SELSTKCIIKTVEFAKQLPGFTTLTIADQITLLKAACLDILIIRICTR--YTP
hTRβ	238	PKFLPEDIGQAPIVNAPEGG-KVDLEAFSHF--TKIITPAITRVVDFAKTLPMFCELPCEDQITLLKGCCMEIMSLRAAVR--YDPE
hVDR	198	DSSSFSMLDLSEEDSDDPSVTLELSQLSMLPHLADLVSYSIQKVIGFAKMIPGFRDLTSEDQIVLLKSSAIEVIMLRSNES--FTMD
cOUP-TF		GYISLILRAKPYPTSRYGSQCMQPNIDMGIENICELAARLLFSAVEWDARNIPFFPDLQITDQVSLLRLTWSELFVLNAAQCSMPLHV
hERR1	294	LVSHLLV-VEPEKLYAMPDPAGPDGHLPAVATLCDLFDREIVVTISWAKSIPGFSSLSLSDQMSVLQSVWMEVLVLGVAQRSIPLQD
hERR2	211	IVSYLLV-AEPDKLYAMPDDVPEGDIKALTTLCDLADRELVFLISWAKHIPGFSNLTLGDQMSLLQSAWMEILILGIVYRSLPYDD
hER	315	MVSALLD-AEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHP-
hGR	531	TLVSLLEVIEPEVLYAGYDSSVPDSTWRIMTTLNMLGGRQVIAAVKWAKAIPGFRNLHLDDQMTLLQYSWMFLMAFALGWRSYRQSS
hMR	737	SPVMVLKNIEPEIVYAGYDSSKPDTAENLLSTLNELGAKQMIQVVKWAKVLPGFRNLPLEDQITLIQYSWMCLSSFALSWRSYKETN
hPR	686	PLINLLMSIEPDVIYAGHDNTKPDTSSSLLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVS

E2

-LDLSQNAVLYGDVMLPQEAFYTS--DSEEMRLVSRIFQTAKSIAEL	DHR3	380	KL-------TETELALYQSLVLLNPE-RNGVRGNTEIQRLF
-YDHSSDSIFFANNRSYTRDSYKN-AGMADNIEDLLHFCRQMFSMKV	EcR	557	----------DNVEYALLTAIV-IFSD-RPGLEKAQLVEAIQ
-FDSSINSIICLN-GQVMRRDAIQ-NGANARFLVDSTFNFAERMNSM	E75A	503	NL-------TDAEIGLFCAIVLITPD-RPGLRNLELIEKMY
-YTPEQDTMTFSDGLTLNRTQMEN-AGFGPLTDLVFAFANQLLPLEM	hRAR	292	----------DDAETGLLSAICLICGD-RQDLEQPDRVDMLQ
-YDPESETLTLNGEMAVIRGQLKN-GGLGVVSDAIFDLGMSLSSFNL	hTRß	361	----------DDTEVALLQAVLLMSSD-RPGLACVERIEKYQ
-FTMDDMSWTCGNQDIKYRVSDVTKAGHSLELIEPLIKFQVGLKKLN	hVDR	325	LE----------EEEHVLLMAICTVSPD-RPGVQDAALIEAIQ
MPLEVAPLLAAAGLHASPMSADRV-VAFTMDE----------IRIFQEN	cOUP-TF		VEKLKALHVDSAEYSCLKAIVLFTSD-ACGLSDAAHIESLQ
LPLQDE--LAFAEDLVLDEEGARA-AGLGEL----------GAALLQL	hERR1	410	VRRLQALRLEREEYVLLKALALANSD------SVHIE-DE
LPYDDK--LAYAEDYIMDEEHSRL-VGLLEL----------YRAILQL	hERR2	328	VRRYKKLKVEKEEFVMLKALALANSD----------SMYIENLE
MEHP-VKLL-FAPNLLLDRNQGKC-VEGMVE---IFDMLLATSSRFR	hER	437	MANLQ-----GEEFVCLKSIILLNSG----------VYTFLSST
YRQSSANLICFAPDLIINE-QRNT-LPCNIDQCKH-----MLYVSSE	hGR	653	LHRLQ---VSYEEYLCMKTLLLLSSVPKDGL----------
YKHTNSQFLYFAPDLVFNE-EKMH-QSMYTELCQG-----MEQISLQ	hMR	859	FVRLQ---TTEEYTIMKVLLLLSTIPKDGL----------
YKHVSCQMLYFAPDLIINE-QRMK-ESSFYSLCLT-----MMQIPQE	hPR	808	FVKLQ---VSQEEFLCMKVLLLLNTIPLEGL----------

E3

DHR3 EIQRLFNLSMNAIRQ----------ELETNHAPLKGDVTVLDTLLNNIPNFRDISILHMESLSKFKLQHPN----VVFPALYKELFS
HcR LVEAIQSYYIDTLRI----------YILNR---HCGDSMSLVFYAKLLSILTELRTLGNQNAEMCFSLKLKNRK------LPKFLEEIWD
E75A LIEKMYSRLKGCLQ----------YIVAQ-----NRPDQFEFLAKLLETMPDLRTLSTLHTEKL----------VVFRTEHKELLR
hRAR RVDMLQEPLLRALKV----------Y-VRK-----RRPSRPHMFPKMLMKITDLRSISAKGAERVITLKMEIPGSM----PPLIQEMLEN
hTRβ RIEKYQDSFLLAFEH----------YINYR-----KHHVTHFMPKLLMKVTDLRMIGACHASRFLHMKVECPTELL---PPLFLEVFED
hVDR LIEAIQDRLSNTLQT----------YIRCRHPPPGSHLLYAKMIQKLA----DLRSLNDDHSKQYRCLSFQ-PEC-SMKLTPLVLEVFGN
cOUP-TF HIESLQEKSQCALEE----------YVRSQ-YPNQPSRFGKLLLRLPSLRTVSSSVIEQLFFVRLVGKTPIE-TLIRDMLLSGSS
hERR1 HIE-DEPRLWSSCE-KLLHEALLEYE----------AGRAGPGGGAERRRAGRLLLTLPLLRQTAGKVLAEFYGVKLNGKVPME-KLFLEMLEAMMD
hERR2 YIENLEAVQKLQ---DLLHEALQDYE----------LSQRHEEEPRRAGKLLLTLPLLRQTAAKAVQHFYSVKLQGKVPME-KLFLEMLEAKV
hER TFLSSTLKSLE----EKDHIHRVLDKITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLY-DLLLEMLDAHRL
hGR --------KSQELFDEIRMTYIKELGK---AIVKREGNSSQNWQRFYQ-LTKLLDSMHEVVENLLN----YCFQTFLD-KTMSIEFPEMLAEIIT
hMR --------KSQAAFEEMRTNYIKELRK---HVTKCPNNSGQSWQRFYQ-LTRLLDSMHDLVSDLLE----FCFYTFRESHALKVEFPAMLVEIIS
hPR --------RSQTQFEEMRSSYIRELIK---AIGLRQKGVVSSSQRFYQ-LTKLLDNLHDLVKQ----LHLYCLNTFIQSRALSVEFPEMMSEVIA

C

	DHR3	EcR	E75A	kni	hRARα	hTRβ	hVDR	cOUP	hERR1	hERR2	hER	hGR	hMR	hPR
DHR3		19	24		18	18	17	14	13	14	15	13	16	15
EcR	55		23		24	29	25	20	16	15	17	14	14	13
E75A	64	55			23	20	22	16	15	15	13	14	12	15
kni	47	48	48											
hRARα	65	55	62	45		33	22	18	18	18	17	14	15	16
hTRβ	56	59	58	52	62		22	16	18	17	19	14	18	15
hVDR	50	58	55	53	47	52		17	20	19	15	13	15	13
cOUP	62	58	58	50	61	59	50		29	30	24	21	19	20
hERR1	48	53	54	43	53	56	45	51		63	32	25	23	25
hERR2	49	51	54	43	54	57	45	54	91		33	25	21	25
hER	53	52	58	52	59	53	47	55	69	72		27	25	27
hGR	47	48	44	45	45	44	41	48	57	57	58		57	54
hMR	52	50	47	47	48	45	44	52	59	59	58	94		56
hPR	48	47	44	45	44	42	42	45	54	54	56	91	91	

WHAT IS CLAIMED IS:

1. An isolated recombinant nucleic acid which, upon expression, is capable of coding for other than a native vertebrate steroid receptor or fragment thereof, said nucleic acid comprising a segment having a sequence substantially homologous to a coding region of domains A, B, D, E or F from an insect steroid receptor superfamily member gene having substantial homology to a steroid binding domain.

2. An isolated recombinant nucleic acid of Claim 1, wherein said insect steroid receptor superfamily member is EcR, DHR3, E75A or E75B.

3. An isolated recombinant nucleic acid of Claim 1, wherein said nucleic acid encodes a polypeptide capable of binding to a ligand for an insect steroid receptor superfamily member.

4. An isolated recombinant nucleic acid of Claim 1, wherein said nucleic acid is capable of hybridizing to an insect steroid receptor superfamily member gene segment under selective hybridization conditions.

5. An isolated recombinant nucleic acid of Claim 4, wherein said selective hybridization conditions are stringent hybridization conditions.

6. A cell transformed with an isolated recombinant nucleic acid of Claim 1.

7. An isolated recombinant nucleic acid having a sequence exhibiting identify over 20 nucleotides of a coding segment of an insect steroid receptor superfamily member having steroid binding domain homology.

8. An isolated recombinant nucleic acid of Claim 7, wherein said nucleic acid encodes a polypeptide which binds to a control element responsive to a ligand of an insect steroid receptor superfamily.

9. A cell transformed with an isolated recombinant nucleic acid of Claim 7.

10. An isolated recombinant nucleic acid comprising a DNA sequence capable of binding to an insect steroid receptor superfamily member other than 20-OH ecdysone receptor.

11. An isolated recombinant nucleic acid of Claim 10, wherein said insect steroid receptor superfamily member is DHR3, E75A or E75B.

12. An isolated recombinant nucleic acid of Claim 10, wherein said DNA sequence promotes transcription of an operably linked sequence in response to binding by said insect steroid receptor superfamily member.

13. An isolated recombinant nucleic acid of Claim 10, wherein said DNA sequence is operably linked to a DNA sequence encoding a polypeptide.

14. An isolated recombinant nucleic acid of Claim 10, wherein said isolated recombinant nucleic acid is an expression vector.

15. A cell transformed with an isolated recombinant nucleic acid of Claim 10.

16. A cell of Claim 15, wherein said cell also comprises said insect steroid receptor superfamily member.

17. A recombinant nucleic acid comprising:

a control element responsive to a ligand of an insect steroid receptor superfamily member ligand responsive control element;

a non-heat shock promoter sequence; and

a sequence comprising a reporter gene.

18. A recombinant nucleic acid of Claim 17, wherein said non-heat shock promoter sequence is an alcohol dehydrogenase promoter.

19. A cell transformed with a recombinant nucleic acid of Claim 17.

20. A cell of Claim 19, wherein said cell is a mammalian cell.

21. A recombinant nucleic acid of Claim 17, comprising additional copies of control lements responsive to a ligand of an insect steroid receptor superfamily member.

22. A method for monitoring expression of a reporter gene comprising the steps of: expressing a recombinant nucleic acid of Claim 17, wherein no radioactive reagent is used in said monitoring of said reporter gene.

23. A polypeptide comprising an insect steroid receptor superfamily member or fragment thereof, wherein said polypeptide is substantially free of naturally-associated insect cell components and exhibits a biological activity characteristic of an insect steroid receptor superfamily member with a steroid binding domain.

24. A polypeptide of Claim 23, wherein said insect steroid receptor superfamily member is selected from the group consisting of EcR, DHR3, E75A and E75B.

25. A polypeptide of Claim 23, wherein said insect steroid receptor superfamily member or fragment thereof also comprises a DNA binding domain.

26. A polypeptide of Claim 23, wherein said insect is Drosophila melanogaster.

27. A polypeptide of Claim 23, which is capable of binding to a hormone analogue selected from the group consisting of an insect hormone and an insect hormone agonist.

28. A polypeptide of Claim 27, wherein said insect hormone is an ecdysteroid.

29. A polypeptide of Claim 27, wherein said insect hormone is 20-OH ecdysone.

30. A polypeptide of Claim 23, which is capable of binding to a DNA control element responsive to an insect horm ne.

31. A polypeptide of Claim 30, wherein said polypeptide comprises a zinc-finger domain.

32. A polypeptide of Claim 30, wherein said insect hormone responsive DNA control element is operably linked to a transcription unit which is responsive to said binding.

33. A polypeptide of Claim 32, wherein said insect hormone responsive DNA control element is upstream from said transcription unit.

34. A polypeptide of Claim 23 fused to a second polypeptide.

35. A polypeptide of Claim 34, wherein said second polypeptide is a heterologous polypeptide.

36. A polypeptide of Claim 35, wherein said heterologous polypeptide comprises a second steroid receptor superfamily member.

37. A polypeptide of Claim 23, wherein said fragment has a sequence substantially homologous to a consensus E1 region sequence.

38. A polypeptide of Claim 23, wherein said fragment has a sequence substantially homologous to a consensus E2 region sequence.

39. A polypeptide of Claim 23, wherein said fragment has a sequence substantially homologous to a consensus E3 region sequence.

40. A polypeptide of Claim 23, wherein said fragment has a sequence comprising:

a segment at least about 25% homologous to a consensus E1 region sequence;

a segment at least about 30% homologous to a consensus E2 region sequence; and

a segment at least about 30% homologous to a consensus E3 region sequence.

41. A composition of matter comprising a polypeptide of Claim 23.

42. A cell comprising a polypeptide of Claim 23.

43. A cell of Claim 42, wherein said cell is a human cell.

44. An antibody or binding fragment thereof exhibiting binding sp cificity to a polypeptide of Claim 23, wherein said binding specificity is directed to an epitope characteristic of an insect steroid receptor superfamily member.

45. A method for selecting DNA sequences capable of being specifically bound by an insect steroid receptor superfamily member, said method comprising the steps of:

screening DNA sequences for binding to a polypeptide of Claim 23; and

selecting said DNA sequences exhibiting said binding.

46. A method of Claim 45, wherein said DNA sequence is operably linked to a gene selected from the group consisting of EcR, DHR3, E74 and E75 genes.

47. A method for selecting ligands specific for binding to a hormone binding domain of an insect steroid receptor superfamily member, said method comprising the steps of:

screening compounds for binding to one or more superfamily members; and

selecting compounds exhibiting specific binding to the members.

48. A method of Claim 47, wherein said ligand is an ecdysteroid.

49. A method of Claim 47, wherein said ligand is a 20-OH ecdysone antagonist.

50. A method for modulating insect physiology or development comprising the steps of:

screening compounds for binding to an insect steroid receptor superfamily member;

selecting said compounds exhibiting said binding; and

administering to an insect said ligand.

51. A m thod of Claim 50, wherein said modulating is lethal to said insect.

52. A fusion polypeptide comprising a hormone binding domain of an insect steroid receptor sup rfamily member and a second polypeptide.

53. A fusion polypeptide of Claim 52, wherein said second polypeptide comprises a DNA binding domain from a second steroid receptor superfamily receptor member.

54. A nucleic acid encoding a fusion polypeptide of Claim 52.

55. A method for selecting ligands specific for binding to a ligand binding domain of an insect steroid receptor superfamily member, said method comprising the steps of:

combining:

(i) a fusion polypeptide of Claim 52, wherein said fusion polypeptide comprises said ligand binding domain functionally linked to a DNA binding domain of a second steroid receptor superfamily member; and

(ii) a second nucleic acid sequence encoding a second polypeptide, wherein expression of said second nucleic acid sequence is responsive to binding by said DNA binding domain; and

screening compounds for an activity of inducing expression of said second polypeptide; and

selecting said compounds.

56. A method of Claim 55, wherein said combining occurs within a cell.

57. A method of Claim 56, wherein said combining step results from expression upon transformation of said cell with a nucleic acid encoding said fusion polypeptide.

58. A method for modulating insect physiology comprising the step of:

administ ring to an insect a ligand sel cted by a method of Claim 55.

59. A method of Claim 55, wherein said binding domain is selected from binding domains of insect steroid receptor superfamily members selected from the group consisting of EcR, DHR3, E75A and E75B.

60. A method of Claim 58, wherein said ligand is lethal to said insect.

61. An isolated receptor control element non-responsive to 20-OH ecdysone comprising a DNA segment capable of binding to an insect steroid receptor superfamily member and capable of controlling expression of an operably linked gene.

62. An isolated receptor control element of Claim 61, wherein said operably linked gene is within about 50 kb of said control element.

63. An isolated receptor control element of Claim 61, wherein said insect steroid receptor superfamily member is DHR3, E75A or E75B.

64. A method for producing a polypeptide comprising the steps of:

selecting a cell which is substantially insensitive to exposure to an insect steroid receptor superfamily ligand;

introducing into said cell:

(i) a receptor for said ligand; and

(ii) a nucleic acid sequence encoding said polypeptide, said nucleic acid sequence operably linked to a control element responsive to presence of said selected ligand, wherein a transformed cell is produced; and

exposing said transformed cell to said ligand.

65. A method of Claim 64, wherein said cell is a mammalian cell.

66. A method of Claim 64, further comprising the step of introducing said cell into an intact organism.

67. A method of Claim 64, wherein said cell is a plant cell.

68. A method of Claim 64, wherein said ligand is 20-OH ecdysone.

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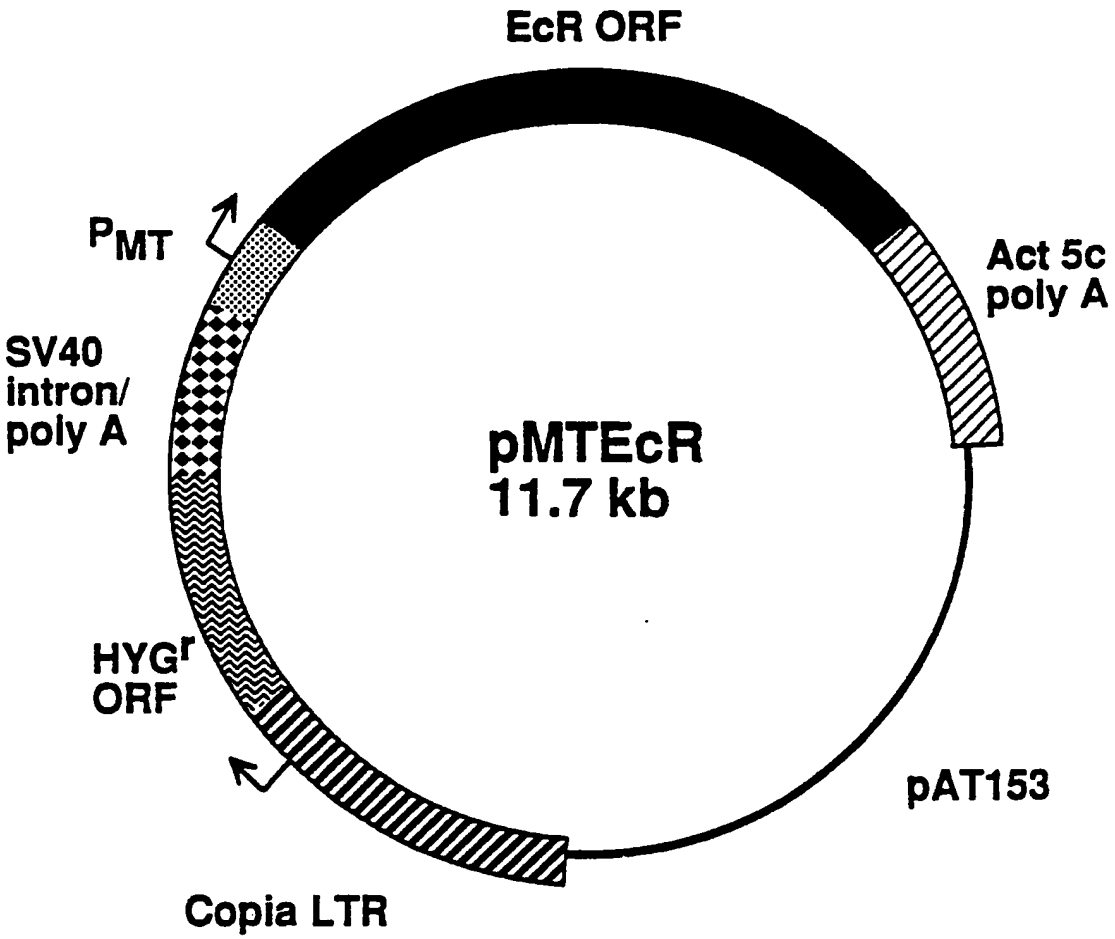
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Copia LTR

FIG._1.

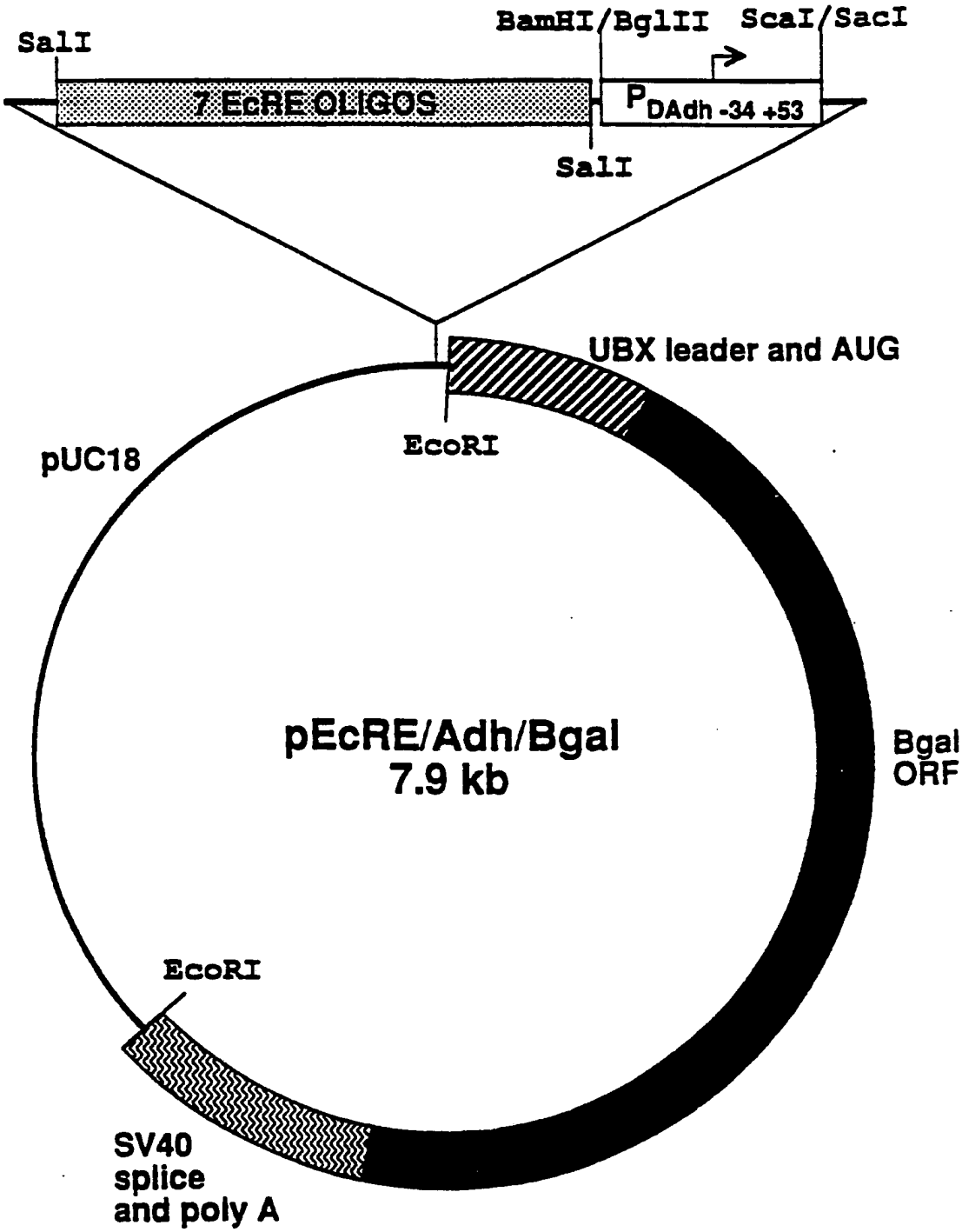

FIG._2.

SUBSTITUTE SHEET

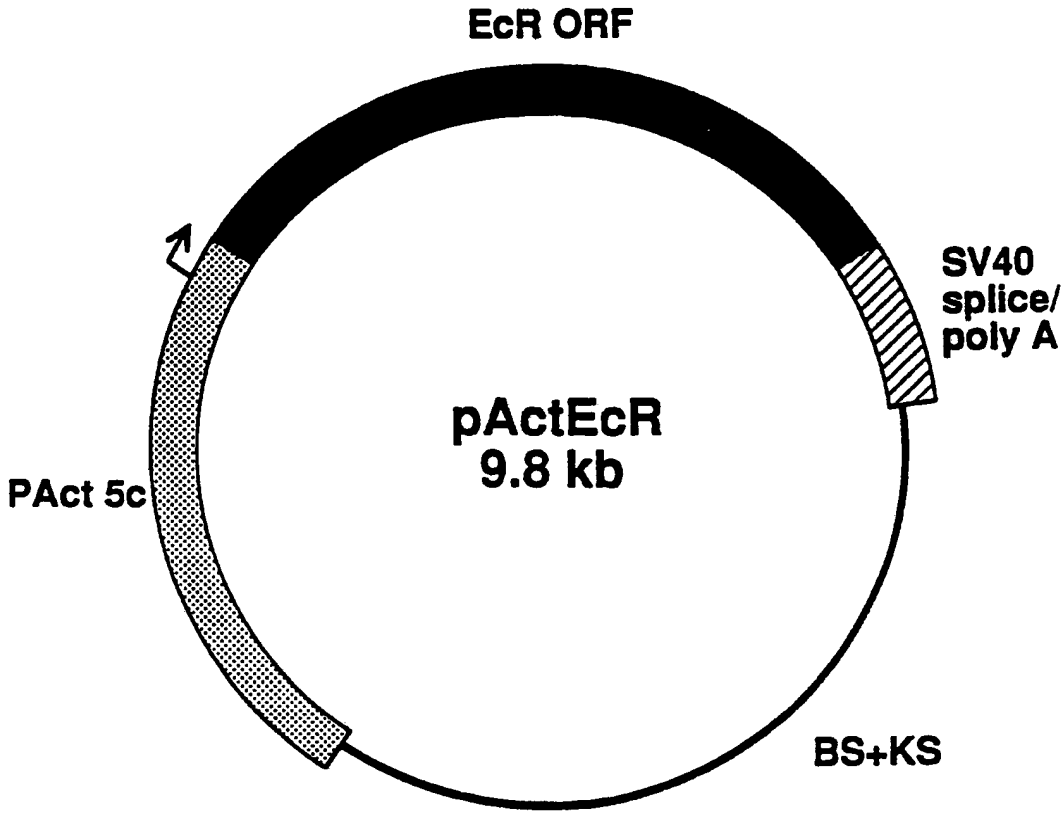

FIG._3.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/01159

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. CL.: 435/69.1; 530/387; 536/27

IPC(5): C12P 21/06; A61K 35/14; C07H 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S. CL.	435/69.1; 530/38.7; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

Chemical Abstracts, Biological Abstracts

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	European Journal of Biochemistry. volume 189. issued 1990. M. Strangmann-Diekmann et al. "Affinity Labelling of Partially Purified Ecdysteroid Receptor with Bromoacetylated 20-OH-ecdysone Derivative". pages 137-143, see pages 138 and 141-143.	23-43
X	Molecular and Cellular Endocrinology. volume 57. issued 1988. M. Lehmann et al. Ecdysteroid Receptors of the blowfly. Calliphora vicina: Partial Purification and Characterization of Ecdysteroid Binding". pages 239-249. see pages 242. 244 and 246-248.	23-43
A	T. Maniatis et al. "Molecular Cloning. A Laboratory Manual" published 1989. Cold Spring Harbor Laboratory Press (N.Y.). see pages 8.60-8.63.	1-5 7.8

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31 May 1991	27 JUN 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Deborah Crouch

Form PCT/ISA/210 (second sheet) (Rev 11-87)

FURTHER INFORM ION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

Claims 1-5, 7-8, 23-43 telephone practice

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

LACK OF UNITY OF INVENTION

PCT/US91/01189

I. Claims 1-5 and 7-8; insect steroid receptor DNA; classified in 536/27

II. Claims 6 and 9; cells transformed with insect steroid receptor DNA; classified in 435/69.1

III. Claims 10-14; DNA sequence binding to insect steroid receptor DNA; classified in 536/27

IV. Claims 15 and 16; cells transformed with DNA sequence binding to insect steroid receptor; classified in 435/69.1

V. Claims 17-21; DNA construct; classified in 536/27

VI. Claim 22; method for monitoring expression; classified in 435/6

VII. Claims 23-43; a polypeptide; classified in 530/350

VIII. Claim 44; antibody; classified in 350/387

IX. Claims 45-49; method to select DNA sequence which binds insect steroid receptor; classified in 435/6

X. Claims 50, 51, 58 and 60; method to modulate insect physiology; classified in 424/405

XI. Claims 52 and 53; fusion polypeptide; classified in 530/350

XII. Claim 54; nucleic acid; classified in 536/27

XIII. Claim 55-57 and 59; method for selecting ligands; classified in 436/501

XIV. Claims 61-63; receptor control elements; classified in 536/27

XV. Claims 64-68; method to produce a polypeptide; classified in 435/69.1

Groups I, II, III, IV, V, VII, VIII, XI, XII and XIV are distinct products. Groups VI, IX, X, XIII and XV are distinct methods. The claims of these groups are drawn to distinct inventions, which are not linked to form a single inventive concept. PCT rules 13.1 and 13.2 does not allow an application to contain more than one inventive concept. All other products, methods of making and methods of using are additional invnetions.